Development of a β-Secretase Activated Prochelator and FRET Probe to Mediate Copper Toxicity in Alzheimer’s Disease

by

Drew S. Folk

Department of Chemistry
Duke University

Date:_______________________

Approved:

___________________________

Katherine J. Franz, Supervisor

___________________________

Qiu Wang

___________________________

Michael C. Fitzgerald

___________________________

Dewey G. McCafferty

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the Graduate School of Duke University

2012
ABSTRACT

Development of a β-Secretase Activated Prochelator and FRET Probe to Mediate Copper Toxicity in Alzheimer’s Disease

by

Drew S. Folk

Department of Chemistry
Duke University

Date:_______________________

Approved:

___________________________
Katherine J. Franz, Supervisor

___________________________
Qiu Wang

___________________________
Michael C. Fitzgerald

___________________________
Dewey G. McCafferty

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the Graduate School of Duke University

2012
Abstract

Alzheimer’s disease (AD) is a progressive neurodegenerative disease that affects over 5 million people in the United States alone. This number is predicted to triple to by the year 2050 due to both increasing life expectancies and the absence of disease-attenuating drugs. The etiology of AD remains unclear, and although there are multiple theories implicating everything from oxidative stress to protein misfolding, misregulated metal ions appear as a common thread in disease pathology.

Chelation therapy has shown some effectiveness in clinical trials, but to date, there are no FDA-approved metal chelators for the treatment of AD. One of the biggest problems with general chelators is their inability to differentiate between the metal ions involved in disease progression verses those involved in normal metabolic function. To address this problem, we have developed a prochelator approach whereby the prochelator (SWH) does not bind metals with significant biological affinity. However, once activated to the chelator (CP) via enzymatic hydrolysis, the molecule is able to bind copper and reduce its toxicity both in vitro and in a cellular model of Alzheimer’s Disease.

Central to this strategy is the site-specificity provided by enzymatic activation of the prochelator. In our system, SWH to CP conversion is mediated by β-secretase, an enzyme involved in Aβ generation. However, in order to render SWH capable of hydrolysis in cells, we modified the prochelator to contain a dihydrocholesterol membrane anchor attached via a polyethylene glycol linker. From this construct, we created β-MAP, which is an SWH-based FRET probe to demonstrate β-secretase-mediated conversion of SWH to CP. β-MAP was also used to verify the efficacy of a known β-secretase inhibitor without the need to for mutated cells lines or expensive
antibodies. The special and temporal assessment of β-secretase activity provided by β-MAP and the associated microscopy method represent a significant advancement to the currently available ELISA assays.

While activation of the prochelator by an enzyme in cells is encouraging, non-specific hydrolysis of the peptide, as demonstrated by β-MAP, prevents significant accumulation of the chelator on the cell membrane. Furthermore, attachment of the polyethylene glycol and sterol units induce cell toxicity not seen with the native CP peptide. These drawbacks prevent the current prochelator from effectively protecting cells from AD conditions. Structural modifications to overcome these problems, including implementation of a new peptide sequence are planned for future experiments.
Contents

Abstract ................................................................................................................................................. iv

List of Tables ........................................................................................................................................... x

List of Figures ......................................................................................................................................... xi

List of Abbreviations ............................................................................................................................. xv

1. Alzheimer’s Disease Progression and Targets of Therapeutic Intervention ................................. 1
   1.1 The Amyloid Cascade Hypothesis ................................................................................................. 1
   1.2 Role of Metal Ions in AD .............................................................................................................. 9
   1.3 Therapeutic Interventions ........................................................................................................... 13

2. A Prochelator Activated by β-Secretase Inhibits Aβ Aggregation and Suppresses Copper-Induced ROS Formation ................................................................................................. 19
   2.1 Background and Significance ...................................................................................................... 19
   2.2 Results and Discussion ................................................................................................................. 25
      2.2.1 Description of Peptides .......................................................................................................... 25
      2.2.2 Enzyme Reactivity: Prochelator-to-chelator conversion by BACE ..................................... 27
         2.2.2.1 LC-MS Assay .................................................................................................................... 27
         2.2.2.2 Calcein Assay .................................................................................................................. 33
      2.2.3 Serum Stability and D-amino acid variants ........................................................................... 35
      2.2.4 SWH and CP copper binding ................................................................................................. 38
         2.2.4.1 CP copper binding ............................................................................................................. 38
         2.2.4.2 SWH copper binding ......................................................................................................... 39
      2.2.5 Copper Transfer from Aβ to CP .............................................................................................. 41
      2.2.6 Protection against hydroxyl radical production: Deoxyribose Assay ............................... 44
      2.2.7 Protection against hydrogen peroxide production: Amplex Red Assay ............................ 46
2.2.8 CP inhibits and reverses Cu-induced Aβ aggregation ........................................ 47
2.3 Conclusions .................................................................................................................. 49
2.4 Experimental ............................................................................................................... 49
  2.4.1 Materials and Instrumentation .............................................................................. 49
  2.4.2 Preparation of Peptides .......................................................................................... 50
  2.4.3 Preparation of Aβ .................................................................................................. 50
  2.4.4 LC-MS Kinetic Assay of BACE Activity ................................................................. 51
    2.4.4.1 LC-MS Assay ................................................................................................. 51
    2.4.4.2 Calcein Assay ............................................................................................... 51
  2.4.5 Serum Stability Assays ......................................................................................... 52
  2.4.6 Copper Binding ..................................................................................................... 53
    2.4.6.1 CP Copper Binding ........................................................................................ 53
    2.4.6.2 SWH Copper Binding .................................................................................... 55
  2.4.7 Copper Transfer from Aβ to CP by Fluorescence Quenching ............................... 56
  2.4.8 Assessment of Aβ Aggregation: Turbidity Assay .................................................. 56
  2.4.9 Amplex Red Assay ............................................................................................... 57
  2.4.10 Deoxyribose Assay ............................................................................................ 58
3. Converting SWH to CP in Living Cells and the Development of β-MAP ..................... 60
  3.1 Background and Significance ..................................................................................... 60
  3.2 Results and Discussion ............................................................................................. 66
    3.2.1 SWH and CP in Cells .......................................................................................... 66
      3.2.1.1 Toxicity and Stability of SWH and CP ......................................................... 66
      3.2.1.2 Cell Supernatant Calcein Assay ................................................................. 68
    3.2.2 Lipid Raft Targeting ............................................................................................ 74
3.2.3 Cholesterol Anchored Prochelators in the Calcein Assay ............................... 78
3.2.4 SWH-FRET Constructs .................................................................................. 81
3.2.5 PEG Linkers .................................................................................................. 86
3.2.6 FPC ................................................................................................................ 87
3.2.7 β-MAP ........................................................................................................... 91
3.2.8 β-MAP in vitro .............................................................................................. 92
3.2.9 β-MAP in HeLa Cells .................................................................................... 93
3.2.10 Quantitation of β-MAP Images .................................................................. 97
3.2.11 β-MAP Specificity for BACE ..................................................................... 98
  3.2.11.1 siRNA ................................................................................................. 98
  3.2.11.2 Broadband Protease Inhibitor Controls ............................................... 99
  3.2.11.3 Scrambled β-MAP ................................................................................. 103
3.2.12 BACE Inhibitor Axon 1125 ........................................................................ 105
3.3 Conclusions ...................................................................................................... 109
3.4 Experimental ..................................................................................................... 110
  3.4.1 Materials and Instrumentation .................................................................. 110
  3.4.2 Preparation of Peptides .............................................................................. 111
  3.4.3 Cell Culture ................................................................................................. 112
  3.4.4 SWH and CP Toxicity ................................................................................. 113
  3.4.5 Cell Supernatant Calcein Assay ................................................................. 114
  3.4.6 Lipid Raft Targeting .................................................................................... 114
  3.4.7 DMACA-DABCYL Characterization ......................................................... 115
  3.4.8 In vitro Assays of BACE Activity ............................................................... 116
  3.4.9 β-MAP Toxicity .......................................................................................... 116
List of Tables

Table 1: BACE substrates evaluated during the development of SWH ........................................... 26
Table 2: D-SWH variants evaluated as BACE substrates ................................................................. 38
Table 3: Stability constants of selected copper chelators ............................................................... 41
Table 4: Serum Stability Assay Parameters ...................................................................................... 53
Table 5: SWH and CP stability ........................................................................................................ 68
Table 6: Protease inhibitors used in specificity studies ................................................................. 100
List of Figures

Figure 1: Aβ Accumulation and Mechanisms of Neurodegeneration in the CNS .......... 4
Figure 2: AD therapeutics ......................................................................................... 18
Figure 3: Strategies toward Aβ-targeted metal chelators ....................................... 23
Figure 4: Prochelator strategy for sequestering copper in AD ............................... 25
Figure 5: SWH Prochelator incubated with BACE for 5 days ............................... 28
Figure 6: Hydrolysis of SWH by BACE ................................................................. 30
Figure 7: Calcein ................................................................................................. 33
Figure 8: Calcein Assay ...................................................................................... 33
Figure 9: CP production monitored via calcein fluorescence ................................. 34
Figure 10: LC Chromatograms of SWH and D-SWH .......................................... 36
Figure 11: Half-life Determination of SWH ............................................................ 37
Figure 12: Titration of CP(Cu) with NTA ............................................................... 39
Figure 13: SWH copper binding study ................................................................. 40
Figure 14: Copper transfer from Aβ to CP ............................................................. 43
Figure 15: Fenton Reaction with copper ............................................................... 44
Figure 16: Protective effects of CP and SWH as measured by the deoxyribose assay .... 45
Figure 17: Amplex red assay measuring hydrogen peroxide production ............... 47
Figure 18: Aβ aggregation assay ......................................................................... 48
Figure 19: Calcein fluorescence quenching from copper chelation ..................... 52
Figure 20: CP(Cu) Extinction Coefficient .............................................................. 54
Figure 21 H₂O₂ Calibration Curve ....................................................................... 58
Figure 22: Deoxyribose Assay .......................................................................... 59
Figure 23: SWH Toxicity in HeLa Cells ................................................................. 67
Figure 49: In vitro enzyme and inhibitor effects on DMACA-SWH-DABCYL .......................................... 101
Figure 50: β-MAP with inhibitors in HeLa cells .................................................................................. 103
Figure 51: Scrambled β-MAP in HeLa cells ...................................................................................... 104
Figure 52: HeLa cells treated with β-MAP and Axon 1125 ................................................................ 106
Figure 53: Progress curves for HeLa cells treated with BACE inhibitor Axon 1125 .......................... 107
Figure 54: Rate of fluorescence turn-on in response to BACE Inhibitor Axon 1125 .......... 108
Figure 55: Optimal HeLa cell plating density in 96-well plates ......................................................... 113
Figure 56: BACE Inhibitors .............................................................................................................. 116
Figure 57: HeLa cell viability in pH 4.5 OptiMEM ........................................................................... 117
Figure 58: Aβ and copper toxicity in HeLa cells ................................................................................. 124
Figure 59: Aβ(Cu) toxicity in undifferentiated SH-SY5Y cells ......................................................... 127
Figure 60: SH-SY5Y cells differentiated in retinoic acid ............................................................... 128
Figure 61: Aβ(Cu) toxicity in differentiated SH-SY5Y cells ............................................................ 129
Figure 62: Protective effects of CP against Aβ(Cu) toxicity in differentiated SH-SY5Y cells ........ 130
Figure 63: Protective effects of substiochiometric CP against Aβ(Cu) toxicity in differentiated SH-SY5Y cells ................................................................................................................. 132
Figure 64: Structure of CPRC ........................................................................................................ 133
Figure 65: CPRC toxicity in differentiated SH-SY5Y cells ............................................................. 134
Figure 66: Protective effects of CPRC against Aβ(Cu) toxicity in differentiated SH-SY5Y cells .. 135
Figure 67: SWH .......................................................................................................................... 142
Figure 68: CP ............................................................................................................................. 143
Figure 69: SW ............................................................................................................................ 144
Figure 70: SW CP ....................................................................................................................... 145
Figure 71: Peptide OK ................................................................................................................... 146
Figure 72: OK-SSH ................................................................. 147
Figure 73: SW-SSH ............................................................... 148
Figure 74: Proteomics 1 .......................................................... 149
Figure 75: Proteomics 2 .......................................................... 150
Figure 76: Proteomics 3 .......................................................... 151
Figure 77: Kyoto ................................................................. 152
Figure 78: Merck ................................................................. 153
Figure 79: F-SWH ................................................................. 154
Figure 80: F-SWH-C ............................................................. 155
Figure 81: C-term ................................................................. 156
Figure 82: N-term ................................................................. 157
Figure 83: MCA-SWH-DNP ................................................... 158
Figure 84: Rho-SWH-Fluor .................................................... 159
Figure 85: DMACA-SWH-DABCYL ....................................... 160
Figure 86: Scrambled FRET ................................................ 161
Figure 87: FPC ................................................................. 162
Figure 88: β-MAP .............................................................. 163
Figure 89: Scrambled β-MAP ............................................... 164
Figure 90: CPRC .............................................................. 165
Figure 91: BACE Inhibitor Axon 1125 .................................... 166
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid-Beta</td>
</tr>
<tr>
<td>ABAD</td>
<td>Amyloid Beta peptide-binding Alcohol Dehydrogenase</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>ATCUN</td>
<td>Amino Terminal Copper and Nickel Binding</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>β-MAP</td>
<td>β-Secretase Membrane-anchored Probe</td>
</tr>
<tr>
<td>BACE</td>
<td>β-Secretase / β-site APP Cleaving Enzyme</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>C99</td>
<td>C-terminal fragment of APP resulting from BACE hydrolysis</td>
</tr>
<tr>
<td>CCS</td>
<td>Metallophaperone for Cu,Zn-Superoxide Dismutase</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan Fluorescent Protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CP</td>
<td>Chelator Peptide / Cleavage Product</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>DABCYL</td>
<td>4-(4-dimethylaminophenylazo)benzoic acid</td>
</tr>
<tr>
<td>DMACA</td>
<td>Dimethylaminocoumarin</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N'-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial Alzheimer’s Disease</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FMOC</td>
<td>9-fluorenlymethoxy-carbonyl</td>
</tr>
<tr>
<td>FRET</td>
<td>Forster (Fluorescence) Resonance Energy Transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GlyGly</td>
<td>Glycylglycine</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen Synthase Kinase 3β</td>
</tr>
<tr>
<td>HBTU</td>
<td>O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
</tr>
<tr>
<td>IDE</td>
<td>Insulin-Degrading Enzyme</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LRP</td>
<td>Lipoprotein Receptor-related Protein</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Media</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular Bodies</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary Tangles</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartic Acid</td>
</tr>
<tr>
<td>NMM</td>
<td>N-methylmorpholine</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PIC</td>
<td>Protease Inhibitor Cocktail</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation End Products</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RARE</td>
<td>Retinoic Acid Response Element</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoic X Receptor</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SOD1</td>
<td>Cu,Zn-Superoxide Dismutase 1</td>
</tr>
<tr>
<td>SWH</td>
<td>Prochelator Peptide</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric Acid</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline and Tween 20</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
</tr>
<tr>
<td>TIS</td>
<td>Triisopropylsilane</td>
</tr>
</tbody>
</table>
Acknowledgements

The list of individuals who deserve my thanks and appreciation for their support during my graduate career is certainly considerable. First and foremost though, I must thank my advisor Dr. Katherine Franz. You allowed me to manage my own projects around my family obligations, which were sometimes extensive, and never once asked me to prioritize lab above all else. Furthermore you gave me the freedom to do research outside the range of the lab’s expertise (and funding), and even when it didn’t look overly promising, you had faith and allowed me to continue. Your influence and advice will certainly guide me throughout my scientific career.

I thank the rest of my committee, Dr. Alvin Crumbliss, Dr. Dewey McCafferty, Dr. Michael Fitzgerald, and Dr. Qiu Wang. Thank you for your commitment, scientific knowledge, and most importantly, unbiased discussions regarding my research projects. It is invaluable to gain advice from experts outside of my own small scientific niche and appreciate not only my work, but also the research of others from a broader perspective.

The members of the Franz lab have contributed both directly and indirectly my successes. Kate Ciesienski and Kathryn Haas probably gave me the hardest time when I joined the lab, but with the benefit of hindsight, I understand why you did and I’m thankful for it. I’m glad that we remain colleagues and friends. I’ll always appreciate Marina Leed (Dickens) for her scientific prowess and compatible sense of humor. Lynne Hyman provided a cool head in tough situations and continues to be a valuable resource even after her graduate career. Jeff Rubino provided a much appreciated dose of peptide knowledge and male influence in a lab dominated by females while Sarah Crider was always there to listen and lend advice when my projects encountered roadblocks. Not to mention she made the best cakes and always had the hook-up for basketball tickets.
Days in lab would have been painfully boring if not for Andy Frank’s singing and quoting of all things Ron Burgundy. Thanks to Marian Helsel for her always-insightful contributions to group meeting. Your progression as a scientist has been fun to watch and I wish you the best in the future. To our newest members; David Besse, Mark Sleeper, Qin Wang, and Qiang Su, thanks for bringing renewed enthusiasm to the lab and good luck as you carve out your own scientific path. I have a special appreciation for the undergraduate students with whom I have been lucky enough to work. Marcus Kaplan and Justin Torosian always kept me moving forward and I hope you learned as much from me as I did from you. Finally, a big thanks to Filip Kielar for providing a seemingly unlimited amount of scientific advice, optimism, and kindness to the lab. I feel very privileged to have worked with you and look forward to hearing about your future successes. There are many others including Sunhee Hwang, Sam Johnson, and Yasheng Gao who have directly contributed to my scientific success, but I do not have space to mention individually. Know that your help has been invaluable.

Most importantly I’d like to thank my family for their support. My wife Amy has been eternally understanding with my occasionally long lab hours and failed experiments, all while pursuing and achieving her law degree and being the best mother and spouse anyone could ask for. No one could maintain the schedule you did with such apparent ease and grace. My children, Nya and Landon, remain my greatest accomplishment and source of greatest joy. Thanks for always reminding me what is really important when the microscope breaks or the HPLC is down. I must also thank my parents and Amy’s parents for their never-ending emotional and financial support.
1. Alzheimer’s Disease Progression and Targets of Therapeutic Intervention

Alzheimer’s disease (AD) is a progressive neurodegenerative disease that affects over 5 million people in the United States. This number is predicted to triple by the year 2050 due to both increasing life expectancies and the absence of disease-attenuating drugs. In addition to the emotional burden placed on patients and caregivers, AD is also a severe economic burden with an estimated annual cost of $604 billion worldwide. In the not too distant future, with an increasing number of patients, AD will overwhelm most health care systems unless better therapeutics are developed. The etiology of AD remains unclear and multiple theories implicating everything from oxidative stress to protein misfolding have been proposed. A common thread between many of these hypotheses is the idea that misregulated metal ions could exacerbate disease pathology. The essential metals copper, iron, and zinc have all been implicated in AD, as well as the non-essential metal aluminum. This chapter describes the prevailing theory of disease progression and how aberrant metal interaction with a variety of biological components could result in neurotoxicity. Additionally, a spotlight on promising AD drugs currently in clinical trials is provided to not only establish the validity of chelation-based therapy, but also to highlight the wide array of approaches being taken to temper this debilitating disease.

1.1 The Amyloid Cascade Hypothesis

The characteristic histology of AD is the presence of insoluble neuritic plaques, intracellular neurofibrillary tangles, degenerating neurons, and activated astrocytes and microglia. While the molecular components leading to these characteristics were unknown at the time, the pathognomonic signs were first identified in 1907 by Alois Alzheimer. Throughout the next 100 years of research since the initial diagnosis,
thousands of papers have been published on AD including those identifying a small 39-43 amino acid peptide, amyloid-β (Aβ), as the main component of the insoluble plaques. Identification of Aβ subsequently spawned the Amyloid Cascade Hypothesis that posits Aβ deposition in brain parenchyma is the initial event in a cascade resulting in the dementia characteristic of AD. This hypothesis combines a wealth of histopathological and genetic evidence that not only explains disease progression, but also identifies targets for possible therapeutic intervention.

The Aβ peptide is synthesized as a constituent of a much larger protein called amyloid precursor protein (APP). The physiological role of APP has not been conclusively identified, though it has been implicated in copper homeostasis and cell signaling. Proteolysis of APP to yield Aβ begins with hydrolysis by an aspartic protease called β-secretase (BACE) in the extracellular domain of APP. BACE cleavage releases a soluble APP fragment to the extracellular environment while the 99-amino acid C-terminus (C99) including the membrane and intracellular domains are retained by the cell. Following BACE cleavage, scission in the membrane domain by γ-secretase liberates the Aβ peptide along with the intracellular C-terminus of APP thought to participate in cell signaling. Genetic mutations in APP and γ-secretase that result in enhanced Aβ production are responsible for many cases of early-onset familial Alzheimer’s Disease (FAD). Even though FAD only accounts for approximately 3.5% of all Alzheimer’s cases, the ability of AD to be an inherited genetic disease lends strong support to the amyloid cascade.

It is important to mention that Aβ production is not the only metabolic fate of APP. In fact, most APP is hydrolyzed in the middle of the Aβ region by α-secretase. This route of hydrolysis precludes Aβ formation and is known as the non-amyloidogenic pathway. Subsequent cleavage by γ-secretase releases a small, non-toxic peptide
fragment called P3 and the intracellular C-terminus. Because either α- or β-cleavage are initial options for APP processing, disruptions in α-secretase activity can direct APP to the BACE-initiated, amyloidogenic pathway and Aβ formation. Thus, stimulation of α-secretase is a sought-after therapeutic strategy for AD.

Aβ production from APP is a ubiquitous and normal metabolic process. In fact, Aβ is found in virtually all biological fluids including the brains of people showing no cognitive dysfunction. Therefore, its accumulation in the brain is likely not only related to its production but also to its propensity to aggregate and be properly cleared from the brain. Aβ clearance is facilitated by a number of enzymes and processes. Proteolytic enzymes such as neprilysin and insulin degrading enzyme degrade Aβ, resulting in a reduction of peptide load. Chaperone molecules such as apoE, lysosomal degradation, and brain export via p-glycoprotein and low-density lipoprotein receptor-related protein (LRP) also contribute to reducing Aβ in the brain. Facilitating Aβ clearance is another pathway for therapeutic intervention, as discussed in later sections.

The neurotoxic processes evoked by Aβ are attracting even more attention as drug targets than Aβ production or clearance. Once Aβ accumulates in the brain, it can interact with a variety of intra- and extracellular processes as shown in Figure 1, leading to neuronal cell death and the outward symptoms of dementia. A brief discussion of some of these mechanisms is outlined below. However, it is important to mention that in addition to direct modes of toxicity, Aβ also influences a number of secondary pathways. For example, Aβ-induced generation of reactive oxygen species can activate c-jun N-terminal kinase, which phosphorylates p66Shc resulting in inactivation of Forkhead transcription factors. While a complete discussion of all of these pathways is outside the range of this chapter, this example underscores the complexity of AD. It also highlights the need to understand the root cause of the disease because targeting these
secondary pathways with pharmaceutics is unlikely to significantly improve patients’ quality of life.

**Figure 1: Aβ Accumulation and Mechanisms of Neurodegeneration in the CNS**

Accumulation of Aβ in the central nervous system (CNS) can occur via a variety of mechanisms including increased neuronal Aβ production, decreased degradation, or imbalances in the import/export mechanisms at the BBB. Soluble Aβ oligomers exert their toxicity by binding to cell surface receptors and altering neuronal signaling cascades as well as catalyzing various forms of oxidative stress. Additionally, oligomers can stimulate microglia to release neurotoxic inflammatory mediators. Mature Aβ plaques also catalyze ROS and can severely damage and interrupt neuronal processes. When produced in stressed neurons, ApoE4 not only enhances Aβ production and inhibits clearance, but can be cleaved into neurotoxic fragments that hinder mitochondrial processes. Tau proteins are implicated in AD pathology by forming large intracellular aggregates and disrupting vital cellular processes. Metals have been found to play a role in virtually all of these phases of AD pathology. Figure reproduced from reference 22.

When aggregated in the extracellular space, Aβ fibrils and plaques induce an immune response from microglia causing inflammation and enhanced localized
oxidative stress. Plaques localized in the synapse also disrupt neuronal signaling and affect long-term potentiation and synaptic plasticity. While this is an important mode of toxicity in AD, the mature plaques are now believed to be a small contributor to disease progression and are more likely a late-stage product of the disease. The primary neurotoxic species are believed to be small oligomeric species of Aβ. These oligomers are intermediary Aβ aggregates between non-toxic monomers and more organized β-sheet fibrils. The exact nature of these oligomers is highly debated in the literature as is their cellular target and mode of toxicity. There are multiple studies on the type and nature of Aβ aggregates and how their reactivity is influenced in various stages of formation. The reader is directed to an excellent review on Aβ oligomers for more information.

In the extracellular domain, soluble Aβ can affect a wide array of processes. Oxidative stress in the form of DNA damage, lipid peroxidation, and protein oxidation is extensive in the AD brain, as is a reduced level of antioxidants such as vitamins E and C, and glutathione. Aβ can catalyze oxidative stress, particularly when complexed with a metal ion such as copper. It is also a moderate oxidant that can generate hydrogen peroxide in the presence of air and ascorbate. Radical formation is particularly damaging in the brain, not only because of the high oxygen metabolism, but also because neuronal membranes contain a relatively high amount of polyunsaturated fatty acids that are particularly susceptible to oxidation. High cholesterol is emerging as a prominent risk factor for AD because statins have been shown to significantly reduce the risk of developing AD. Aβ is able to bind to cholesterol at the cell membrane and oxidize it to 7β-hydroxycholesterol, which is a highly neurotoxic and proapoptotic product.
Aβ can form pores in endothelial cell membranes, which can have extreme consequences at the blood-brain barrier (BBB).\textsuperscript{28} There is ample evidence of BBB damage and intracranial hemorrhage in AD, and whether this is a causative feature of the disease or the cumulative effect of long standing insult, Aβ is thought to play a central role in BBB deterioration.\textsuperscript{29} Perhaps the most detrimental consequence of pore formation is the loss of calcium homostasis in neurons. Calcium is critical for neuronal transmission, and because of the strong concentration gradient maintained at the cell membrane, pore formation and resultant influx of calcium induces excitotoxicity and cell death.\textsuperscript{30} Even if they are not pore-forming, Aβ oligomers simply binding to synaptic membranes interferes with cell signaling and can attenuate synaptic plasticity.

ApoE is a major risk factor for AD. These protein isoforms bind Aβ and facilitate influx into neurons through the low-density LRP receptor.\textsuperscript{31} ApoE also promotes Aβ aggregation into its most toxic oligomeric form\textsuperscript{32} and promotes enhanced Aβ production through regulation of APP recycling.\textsuperscript{33}

In addition to using cell surface receptors such as LRP to gain entry into the cell, Aβ can directly activate receptors to influence a host of cell signaling events. Aβ is capable of interfering with NMDA glutamate receptors and L voltage-sensitive calcium channels inducing oxidative stress and excitotoxicity.\textsuperscript{34} It can also disrupt signal transduction by neuronal insulin receptors, establishing a molecular link between AD and Type II diabetes.\textsuperscript{35}

In addition in extracellular deposits, Aβ can accumulate intracellularly by at least four different pathways: 1) If generated in the endoplasmic reticulum, Aβ could be recognized as a misfolded protein and excreted to the cytosol for proteosome degradation. 2) If generated in the endosome/lysosome system, Aβ increases membrane permeability and could leak into the cytosol. 3) Extracellular Aβ could passively diffuse
through the membrane, or 4) be taken up directly by surface receptors such as α7 nicotinic acetylcholine receptor.\textsuperscript{36} Once present in the cytosol, Aβ seems to be more toxic and more cell specific than extracellular Aβ. Direct microinjection of Aβ into cells induces significant (50-70\%) cell death in primary human neurons but not in astrocytes, NT2, LaN1, or M17 cells.\textsuperscript{37}

Peptide aggregation could exert its toxic effects intracellularly via multiple pathways. It may adversely impact sub-cellular compartments such as inducing swelling of axons or impairing multivesicular bodies (MVB) sorting pathways, which would inhibit proteosome and deubiquitinating enzymes.\textsuperscript{38} Binding to and disrupting the function of protein partners is another means of intracellular toxicity. Aβ interactions with the p53 promoter activates p53, which ultimately results in execution of the cell death pathway.\textsuperscript{39} It can also suppress Akt signaling resulting in glycogen synthase kinase 3β (GSK-3β) activation and cell death.\textsuperscript{40} Aβ can bind to amyloid beta peptide-binding alcohol dehydrogenase (ABAD) and disrupt mitochondrial function whereby promoting reactive oxygen species (ROS) and oxidative stress.\textsuperscript{41} It can bind to catalase and prevent the dismutation of H\textsubscript{2}O\textsubscript{2}, which also contributes to oxidative stress.\textsuperscript{42}

However, the most popular of Aβ’s binding partners is the tau protein. Aβ-binding causes rapid dissociation of tau from microtubules, where it is responsible for maintaining axonal structure.\textsuperscript{43} Without tau, the axon collapses causing malfunctions in the synapse and ultimately cell death. Tau phosphorylation by GSK-3β also induces dissociation and aggregation into neurofibrillary tangles (NFTs).\textsuperscript{44} Both Aβ and ApoE activate GSK-3β and contribute to tau phosphorylation. It has been noted that, unlike Aβ, there is a statistically significant correlation between neuron loss, cognitive decline, and the presence of NFTs.\textsuperscript{45} Studies of frontotemporal lobe dementia indicate that NFTs are directly responsible for the neuronal loss and brain atrophy associated with
In AD however, there is no correlation between Aβ-load and NFTs, and importantly, the amount of neuronal loss greatly exceeds that of NFTs. These observations suggest that NFTs are an end-stage of tau pathology, and the majority of neurons are already lost before the presentation of NFTs. The most accommodating hypothesis that combines both the amyloid cascade and the clinical evidence for tau pathology, involves the initial deposition of Aβ aggregates, which in turn triggers tau hyperphosphorylation and subsequent neuronal loss. The “how” and “why” of this hypothesis remains to be conclusively elucidated, but nonetheless, tau pathology is an important factor in AD etiology.

Intracellular Aβ is not abundant in non-AD neurons, but interestingly, it is even less prevalent in AD neurons with pathologically confirmed amyloid deposition. Extracellular Aβ is also a poor indicator of mental health because plaque load does not correlate with cognitive decline as exemplified by multiple examples of normal individuals with a heavy plaque load. In fact, an Aβ decrease in the cerebrospinal fluid (CSF) is a signature biomarker of AD and some have ascribed this pathological phenotype to the deposition of formerly soluble Aβ into insoluble plaques. Furthermore, in vitro studies show that Aβ is not consistently toxic to neurons until dosed at non-physiological micromolar levels. Therefore, while the amount of evidence indicting Aβ is certainly vast, clinical observations do not condemn the peptide as the only facilitator of disease. These observations are also the primary criticism of the amyloid cascade and suggest that AD is an even more complex disease and involves more molecular players than originally implicated. One such player, metal ions, fill in many of the holes associated with the amyloid cascade and are discussed in more detail below.
1.2 Role of Metal Ions in AD

Metal ions directly participate in AD progression in a variety of ways, including regulation of APP gene expression and localization, determination of how APP is processed by the secretases, and the propensity of Aβ to aggregate and catalyze oxidative stress reactions. Additionally, aberrant metal ions can have indirect causative roles in AD such as interfering in normal protein function and enhancing excitotoxicity.

There are multiple ways in which metals can affect APP and its processing enzymes. The precise function of APP is unclear, though it has been proposed to be a copper chaperone because of its multiple metal binding sites both at the N-terminus of the ectodomain and within the Aβ region. These sites are composed of histidine, tyrosine, and methionine ligands, consistent with the structures of other known copper transporters. Furthermore, APP is sensitive to copper levels, being either up- or down-regulated in response to copper overload or depletion, respectively. Copper also affects the localization of APP within the cell. Studies using SH-SY5Y neuronal cells have shown that copper promotes the exocytosis of APP at the cell membrane while concurrently inhibiting its endocytosis. Presumably, this exocytosis makes APP more available for amyloidogenic processing by BACE, which is located at the luminal membrane. Additionally, BACE itself contains a copper atom in its C-terminal domain, which it acquires from the copper chaperone CCS. Because CCS is unable to properly deliver copper to its protein target superoxide dismutase (SOD1) under conditions of high BACE expression, the cell’s primary defense against ROS becomes weakened, and the cell is subject to increased oxidative damage.

---

1 Republished in part from Folk, D. S.; Kielar, F.; Franz, K. J. 3.15 Bioinorganic Neurochemistry Comprehensive Inorganic Chemistry II. Accepted 2012
In addition to copper, zinc may also play a role in APP processing. Zn\(^{2+}\) can bind to APP at multiple sites, the best-characterized being between two adjacent cysteine residues.\(^{50}\) Zinc binding at this site can affect the protein’s dimerization and aggregation. There is also a zinc-binding site in the A\(\beta\) region of APP that spans the \(\alpha\)-secretase cleavage site.\(^{55}\) Therefore, under conditions of elevated zinc, \(\alpha\)-secretase may be inhibited, encouraging amyloidogenic processing of APP. Conversely, because \(\alpha\)-secretase contains a zinc atom in its catalytic domain, low zinc levels or mutations to zinc binding site residues can also inhibit activity.\(^{56}\) Abnormal zinc homeostasis can also affect the A\(\beta\)-degrading enzymes insulin-degrading enzyme (IDE) and neprilysin, which are both zinc-containing metalloproteases. These factors highlight how even slight misregulations in copper and zinc levels can significantly contribute to AD pathology.

Iron is also involved in APP regulation. APP translation is regulated by an iron response element located in the 5’ region of its associated mRNA.\(^{57}\) Excess intracellular iron concentrations selectively up-regulate APP transcription, whereas iron chelation induces a down-regulation of APP.\(^{57}\)

While it is clear that metal ions and metalloproteins participate in the formation and excretion of A\(\beta\), metal ions can also interact with the peptide in the intra- and extracellular space. Metal binding to A\(\beta\) can have numerous neurotoxic effects. Zinc and, to a lesser extent, copper and iron have been shown to exacerbate A\(\beta\) aggregate formation.\(^{58}\) The concentrations of these metals needed to elicit this effect are reported to be in the low micromolar range. While these conditions are nearly impossible to achieve in the periphery due to the tight regulation of these metals both intracellularly (via chaperones, transporters, and storage proteins) and extracellularly (via human serum albumin, transferrin, and ceruloplasmin), the brain has unique features that may
permit these concentrations of free metals to be available. While the concentration of human serum albumin (HSA) in the blood is 600 µM, HSA is only present in CSF up to 3 µM and, thus, is not as effective at sequestering excess metals.\textsuperscript{59} Even more significant, perhaps, may be the observations that particular neurons can release transiently high levels of metal ions during synaptic transmission. Synaptic zinc is the best characterized, while copper, iron and manganese have all been proposed as synaptic modulators, but await further experimental vetting. This release of metal ions may account for the synaptic localization of Aβ aggregates and the observation of copper and zinc being highly enriched in Aβ plaques. It may also explain why the areas of the brain that are most affected by AD contain the highest amount of metal-releasing neurons.\textsuperscript{52}

In addition to causing protein precipitation, metals bound to Aβ may also promote oxidative stress. Much of the damage observed in the post-mortem AD brain can be ascribed to oxidative damage; thus, preventing oxidative damage is a central focus in AD research.\textsuperscript{60} In the presence of oxygen and biological reducing agents such as ascorbate, Cu-Aβ has been shown to undergo redox chemistry to generate hydrogen peroxide and ultimately hydroxyl radicals via Fenton chemistry.\textsuperscript{61} These ROS can then proceed to react with biomolecules to generate oxidative products that have been found extensively in the brains of AD patients: lipid peroxidation; DNA, RNA, and protein oxidation; advanced glycation; and nitration.\textsuperscript{60} Oxidation of Aβ itself can also occur, which results in dityrosine crosslinked oligomers.\textsuperscript{61} These species are not only highly toxic but are also stable to proteolysis and thus resist degradation. Additionally, there are significantly reduced levels of antioxidants such as vitamin E and glutathione in the AD brain. Oxidative damage to cell membranes and DNA can either kill neurons directly or initiate a pathway within the cell that ultimately leads to apoptosis.\textsuperscript{20}
Cholesterol plays an important role in the brain and in the toxicity associated with metal-Aβ species. Cholesterol is used extensively in the synthesis of myelin, which is used to insulate neuronal axons and support transmission. Additionally, cholesterol is a critical component of neuronal cell membranes where it is involved in sorting membrane proteins and receptors. Cholesterol can bind to Cu-Aβ complexes and be oxidized to 7β-hydroxycholesterol with concomitant production of hydrogen peroxide. This 7β-hydroxycholesterol is a signaling molecule that initiates apoptosis and contributes to neuronal cell death. Additionally, cholesterol-rich microdomains known as lipid rafts not only coordinate the binding of Aβ to cell membranes and enhance toxicity but also facilitate amyloidogenic processing of APP by colocalizing BACE and APP.

The observation of a high deposition of copper and zinc in amyloid plaques has bred many theories about the involvement of these metals in AD. The role of misregulated iron is less well established because iron is not as enriched in AD plaques. However, a few pathways have been identified in addition to iron’s role in APP translation. The mitochondria, which heavily utilize iron in oxygen metabolism, are altered in AD. This alteration not only results in defective ATP production and increased oxidative stress but also limits the cell’s ability to protect itself from these insults. Aβ is capable of binding and reducing iron so the metal can initiate oxidative stress reactions analogous to those catalyzed by copper. Similarly, redox-active iron is found in NFTs composed of hyperphosphorylated tau protein.

In addition to the direct evidence for iron-related damage, there is evidence that some iron proteins are misregulated in AD. The iron cores of ferritin were observed to be different in AD patients than in physiological controls because they do not contain ferrihydrite or hematite. Lactoferrin, an iron binding protein, also shows abnormal
homeostasis in AD such that it selectively accumulates in senile plaques and NFTs. These latter two observations, while not directly implicating iron in AD pathology, may have implications for disease progression and early diagnosis.  

Finally, aluminum has been implicated in AD pathology for many years. Theories began with studies that correlated cognitive impairment in men to the high aluminum exposure at their work environment. Subsequent studies supported this theory by showing the occurrence of Alzheimer’s Disease was slightly higher (1.5-fold) in areas where the water supply contained elevated levels of aluminum.  

It was assumed that aluminum can hijack iron transport machinery and enter the brain; however, more recent studies have shown that there is virtually no difference in the aluminum concentration in AD and healthy brains. Furthermore, because aluminum has access to only one relevant valence state (+3), it is unable to be reduced and cannot be released from transferrin for incorporation into the cytosolic proteins. Thus, the implications of aluminum in AD pathology have been largely curtailed.  

1.3 Therapeutic Interventions

Even though AD has been studied for over 100 years, there are only five FDA approved therapeutics to treat the disease. Four of them, Razadyne® (galantamine), Exelon® (rivastigmine), Aricept® (donepezil), and the rarely prescribed Cognex® (tacrine) are cholinesterase inhibitors. Because cholinergic neurons of the hippocampus are strongly affected in AD, the neurotransmitter acetylcholine, which is responsible for learning and memory, becomes highly depleted in affected brains. Cholinesterase inhibitors are presumed to prevent the breakdown of existing acetylcholine, thus preserving higher amounts of the neurotransmitter in the brain. Due to their mechanism of action, these treatments are mildly effective in delaying AD symptom progression, but the effects only last for 3 to 6 months, after which, these
drugs are ineffective. The fifth compound, Namenda® (memantine) is an NMDA antagonist and is the only drug approved for moderate to severe AD. Memantine works by preventing excessive glutamate from interacting with the NMDA receptor and inducing excitotoxicity. Similar to the cholinesterase inhibitors, the effects of memantine are modest and transient. Combination therapies that utilize both memantine and a cholinesterase inhibitor are often more effective than a single therapy, but still only delay symptom progression by a few months. All five of these drugs target very late-stage effects of AD and cannot prevent, stop, or reverse AD progression. Thus, society is in desperate need of therapeutics with more up-stream targets. Fortunately, due to steady increases in our understanding of AD combined with better diagnostic technology, there are a number of drugs in clinical trials that target various pathways in the amyloid cascade. If nothing else, the next decade will reveal whether or not the Amyloid Cascade Hypothesis is on the right track, or whether we need to explore alternative theories.

There are three main areas in which to intervene directly in the amyloid cascade: 1) reducing Aβ production, 2) preventing Aβ aggregation, and 3) enhancing Aβ clearance. In targeting Aβ production, the most obvious targets are β- and γ-secretase. There are multiple reports of BACE inhibitors in the literature and some are in early stage trials. However, progress in this area has been slow due the difficulty in developing drug-like, blood brain barrier (BBB) permeable molecules that can effectively and selectively inhibit BACE’s large active site. A more indirect approach to inhibiting BACE hydrolysis is to prevent the enzyme from co-localizing with APP in early endosomes by interfering with the small GTPase ARF6. This approach has yet to be validated and no drugs of this type are in trials.
The other enzyme involved in Aβ production is γ-secretase and accordingly, there are several reported compounds that inhibit or modulate this enzyme. However, γ-secretase is also proving to be a difficult target. A phase III clinical trial of the γ-secretase modulator, tarenflurbil, was found to be ineffective and even worse, a phase III trial with Eli Lilly’s compound, semagacestat, was halted because it actually accelerated cognitive decline and several patients developed skin cancer. More recent evidence suggests that γ-secretase is a critical component in the Notch signaling pathway and thus permanent inhibition is not an option for therapeutics.

Bristol-Meyers Squibb developed a compound, BMS-708163, with an alternative mode of action as it is designed to prevent formation of the mature γ-secretase cluster (4 enzymes). This drug demonstrated moderate efficacy in phase II trials and phase III trials are planned. Persuing other modulators of γ-secretase that selectively prevent APP hydrolysis while allowing Notch signaling continues to be an attractive therapeutic option.

Another approach to therapeutics is to target Aβ directly. Elan’s scyllo-inositol is designed to prevent Aβ aggregation but did not show efficacy in a phase II clinical trial; however, phase III planning at a higher dose is ongoing. Directly relevant to our work, a small phase IIa clinical trial with the metal chelator PBT2 has shown preliminary promise, presumably by reallocating misregulated metal ions associated with Aβ deposits. Another approach towards directly targeting Aβ utilizes the tricyclic pyrone, CP2 and its analogues, which can reduce toxicity and Aβ oligomerization in neuroblastoma MC65 cells and primary cortical neurons. CP2 is interesting because it is reported to selectively target intracellular Aβ without altering extracellular Aβ levels. CP2 is one of a number of Aβ targeting compounds with in vitro efficacy reported in the literature; however, only a few have progressed to clinical trials.
A third approach in targeting the amyloid cascade is to facilitate Aβ clearance from the brain. Treatment with anti-Aβ antibodies in the periphery is a creative and intriguing pathway to reduce Aβ load. By binding Aβ in the blood, the antibodies act as a peripheral sink creating an Aβ deficiency in the blood. This deficiency disrupts the Aβ equilibrium that exists between the blood and brain and facilitates export of Aβ from the brain. Alternatively, antibody binding to mature plaques activates microglia that phagocytose the aggregates and export them from the brain. There are several phase III clinical trials currently ongoing that implement this strategy. Pfizer/Janssen’s compound, Bapineuzumab and Eli Lilly’s Solanezumab are both humanized monoclonal antibodies, which target the N-terminus and center of the Aβ peptide, respectively. Cautious optimism surrounds these trials. Although both have demonstrated the ability to reduce the Aβ load in the brain during phase II trials, neither greatly improved the cognitive ability of the patients. Similar results were seen with vaccination treatments that activate the body’s own antibody system against Aβ, leading many to believe that either facilitating Aβ clearance alone is not enough or that the damage has already been done and this type of treatment would only be effective for early stage AD.

Tau is also an interesting target in AD, and is starting to receive more attention given the difficulties and disappointments with targeting Aβ-pathology. A phase II trial conducted by TauRx Pharmaceuticals in Singapore with methylene blue (Rember) showed promising results by slowing disease progression in 81% of patients. Currently, phase III trials with a newer formulation, LMTX are planned. Allon’s compound, Davenutide, which works by preventing tangle formation, recently completed phase II trials for AD-like mild cognitive impairment with promising results. However, it is being redirected for the treatment of frontotemporal dementia given its tau-specific target. Another popular intervention into tau pathology involves inhibiting GSK-3β
and preventing tau phosphorylation. Noscira has synthesized a compound called Nypta undergoing a phase IIb clinical trial after phase IIa reported positive results in 4 out of 5 groups tested.\textsuperscript{75}

Several other strategies involving more indirect targets for AD have also progressed to clinical trials. RAGE is a receptor capable of transcytosing Aβ across the BBB from the blood into the brain. Inhibition of RAGE is an attractive therapeutic option because it has been found to not only facilitate Aβ entry into the brain but also induce oxidative stress and inflammatory responses characteristic of AD.\textsuperscript{80} Pfizer’s RAGE inhibitor, PF\textsubscript{0}4494700, was in clinical trials until late 2011 until it was discontinued because no benefits were seen. Ceregene is exploring enhancement of the brain’s own defensive mechanisms by directly implanting nerve growth factor genes.\textsuperscript{75} The phase II trial is currently ongoing; however, this approach requires surgery and may not be the most attractive therapeutic option. Other therapeutics such as antioxidants, anti-inflammatory drugs, or mitochondrial protectors such as Pfizer’s phase III drug, Latrepirdine, have shown limited effects so far. A sample of the potential AD therapeutics discussed above is given in Figure 2 with the molecular target of each drug listed in parenthesis.
There are a vast number of prospective mechanisms involved in AD progression and with them, a huge number of potential targets for pharmaceutical intervention. Many of these targets already proved to be dead ends (γ-secretase inhibition) or yielded disappointing results (RAGE); however, multiple avenues still remain (chelation). There is equal reason for optimism and despair within the field of AD therapeutics. The more we think we know about AD, the more we’re proven incorrect. Perhaps a combination therapy or a single drug with multiple targets will prove to be the most effective treatment for AD.

To this end, we envisaged a specific metal chelator capable of interrupting an array of deleterious downstream pathways. We hypothesize that selective localization of the chelator to pre-established targets in the amyloid cascade will not only enhance the efficacy of chelation therapy already being demonstrated by PBT2 but also improve the toxicity profile. The following chapters describe our in vitro and cellular results towards designing a chelator with these properties.
2. A Prochelator Activated by β-Secretase Inhibits Aβ Aggregation and Suppresses Copper-Induced ROS Formation

Among the many impaired processes in AD, copper dishomeostasis represents an area that could be responsible for multiple neurotoxic pathways in disease progression. Our approach to potential therapeutic intervention involves trying to prevent and/or reverse Aβ metalation in an attempt to mediate aberrant metal toxicity. Chelation must be done site-specifically, however, because while stripping Aβ of copper may be beneficial, removing copper from essential cuproproteins could result in deleterious off-target effects. To accomplish site-specific chelation, we envisaged a prochelator approach that would mask the metal-binding site of a chelating molecule until acted on by a stimulus, preferably at the site of Aβ-Cu interaction. The following sections describe the in vitro results towards developing a prochelator, which is activated by an enzyme involved in Aβ generation, to yield a chelator capable of attenuating copper’s reactivity.

2.1 Background and Significance

There is an overwhelming amount of genetic and histopathological evidence that implicates the Aβ peptide in AD pathogenesis. These studies gave rise to the Amyloid Cascade Hypothesis of disease progression, which posits that downstream effects and symptoms of Alzheimer’s begin with excess accumulation and deposition of Aβ in the brain. However, several unexplainable anomalies arise from indicting Aβ and resultant plaque formation as the only facilitators of disease. First among them is the ubiquitous nature of the peptide. Aβ is a normal metabolic product, and the peptide is found in the

---

blood, CSF, and brains of healthy people and AD patients alike.\textsuperscript{82} Even more perplexing is in vitro data that suggest A\textbeta{} is only toxic to neurons at micromolar concentrations, and in fact, at physiologically relevant nanomolar concentrations, A\textbeta{} is actually neuroprotective.\textsuperscript{83}

Why then, does A\textbeta{} aggregate into mature plaques only in certain parts of the brain if it is present in most all biological fluid, and is plaque load a good indication of disease? The lack of plaque formation in the blood can be explained by the high concentration of human serum albumin, which binds the peptide with moderate affinity.\textsuperscript{59} Albumin is present in much lower concentrations in the brain furthermore, the slow self-aggregating properties of A\textbeta{} combined with its low concentration suggest that the presence of A\textbeta{} alone is not enough to induce plaque formation. Ultimately, even plaques themselves are dubious indicators of AD as postmortem examinations of patients showing no ante-mortem symptoms of dementia have reported substantial amounts of amyloid plaques.\textsuperscript{84} Therefore, the presence of A\textbeta{}, either in a soluble or insoluble form, does not seem to be enough on its own to induce AD symptoms.

Ashley Bush and colleagues have coined the “Metal Hypothesis of Alzheimer’s Disease” which acts as an addendum to the Amyloid Cascade Hypothesis. Their theory suggests that even though A\textbeta{} is central to AD progression, it is metal ions, particularly copper and zinc, that regulate A\textbeta{} toxicity, and tip the balance from a harmless metabolic product to neurotoxic agent.\textsuperscript{52} The role of metals in AD fills several holes with the Amyloid Cascade and in our view, represents a more complete explanation of disease etiology.

The aim for pharmaceutical agents based on the Metal Hypothesis is to target a metal ion and sequester it from the amyloid cascade. Copper is a more attractive metal ion target than zinc for several reasons. Even though zinc exacerbates A\textbeta{} fibril formation
more than copper and is found in higher concentrations in AD plaques, it is a poor target for AD treatment because of the anticipated side effects associated with zinc chelation. Zinc is a known neurotransmitter and although its release into the synapse is suspected to play an important role in plaque formation, depriving the brain of signaling molecules while trying to treat AD is counterproductive. Additionally, two of the primary enzymes that degrade Aβ, insulin-degrading enzyme and neprilysin, are both zinc metallopeptidases. Removing zinc from these enzymes would result in a net increase in Aβ. Finally, plaque accumulation is not the primary indicator of brain health. A greater source of neurotoxicity in AD stems from increased oxidative stress and the presence of reactive oxygen species. Zn(II) is a d10, redox-inert metal and, unlike copper, does not participate in Fenton-like chemistry to produce hydroxyl radicals or can be reduced to generate H2O2.

Copper-attenuating drugs represent better lead compounds for AD treatment. General metal chelators such as desferrioxamine and clioquinol have shown promise in preclinical AD studies by presumably stripping metals from Aβ and limiting their ability to catalyze oxidative damage. These ligands have never made it past clinical studies for AD for a variety of reasons, and later generation chelators such as PBT2 are still in clinical trials. General chelators all suffer from the inability to effectively differentiate between different metal ions, and even worse, between the harmful metals associated with the amyloid plaques and essential metals necessary for cell function. For this reason, the development of a chelator with the ability to specifically target copper in Aβ plaques would be highly significant.

Specifically targeting copper in Aβ has been attempted using a number of different strategies. Perhaps the most obvious is to tether an Aβ targeting molecule to a copper chelator. This approach is exemplified by W. Wu and coworkers who reported
tagging the copper chelator apocyclen to Aβ targeting molecules KLVFF and curcumin and observing a decreased toxicity of Aβ(Cu) in cell culture.88 This approach has the advantage of retaining virtually all of the activity of both molecules if the tether is placed in the correct location. Unfortunately, tethered molecules tend to be larger than 500 MW, which makes them unlikely to penetrate the blood brain barrier. To overcome this limitation, the chelator and recognition molecules can be merged to yield hybrid bifunctional molecules.89 Aβ aggregate markers such as thioflavin T or stilbenes are commonly used as targeting molecules and are merged with clioquinol or iminopyridines as the copper chelators. Particular examples of this hybrid strategy come from Mi Hee Lim’s group in Michigan and their array of compounds, which inhibit Aβ aggregation and reduce metal-related toxicity.90,91 A diagram depicting both of these strategies and relevant examples is presented in Figure 3.
Targeting metal chelators to Aβ with bifunctional molecules. Examples include XH1,\textsuperscript{92} Cyc-KLVFF,\textsuperscript{88} and Cyc-Curcumin\textsuperscript{88} which use the linkage approach to tether a chelating molecule to an Aβ-targeting moiety. An alternative approach is to directly incorporate the chelator (circled) into the Aβ-targeting molecule as demonstrated by HBTI,\textsuperscript{93} IMPY derivative,\textsuperscript{91} and Silbene derivative.\textsuperscript{91}

The Franz Lab at Duke University is responsible for pioneering a third approach to targeting metals in AD. By using a prochelator strategy, the chelating molecule is masked until activated to the chelator by disease conditions. This has a similar site-directing effect as the Aβ-targeted chelators. Marina Dickens has developed QBP, which is a clioquinol-type chelator masked by a boronic ester to prevent metal binding.\textsuperscript{94}
Oxidative stress, which is prevalent in AD, unmasks the boronic ester to yield a chelator capable of sequestering copper from Aβ and limiting future radical production.

We sought a complementary prochelator approach towards site-directing a copper-specific chelator to Aβ(Cu) interactions. Instead of using oxidative stress, we opted for an enzyme critical to AD progression to activate our prochelator. β-Secretase (BACE) is the enzyme responsible for initiating the amyloidogenic processing of APP to yield Aβ. As this event is central to AD pathology, we hypothesize that a prochelator activated at the site of Aβ generation would have enhanced efficacy and specificity over other targeting approaches. Additionally, substrate competition for BACE from a prochelator could also potentially slow down Aβ generation in affected neurons.

Here, we present a peptide substrate of BACE (SWH) that is cleaved by the enzyme to release a copper-specific chelator (CP). CP is specifically modeled after the amino terminal copper and nickel-binding (ATCUN) motif present in human serum albumin, which relies on a free amine terminus to possess potent copper binding abilities. The masking strategy is achieved though incorporation of that free amine into an amide bond in the intact prochelator SWH. Whereas SWH is a very weak copper ligand, CP has strong enough copper binding characteristics to strip the copper from Aβ and solubilize the aggregates characteristic of Alzheimer’s disease (Figure 4). Additionally, removing copper ions from Aβ prevents the oxidative stress reactions promoted by aberrant copper-Aβ complexes implicated in disease etiology.
Results and Discussion

2.2.1 Description of Peptides

The prochelator peptide, termed SWH, has the sequence E-V-N-L-D-A-H-F-W-A-D-R. The first eight amino acids are responsible for enzyme recognition and are based on residues 668–675 of the so-called Swedish mutant APP, which has the sequence EVNLDAEF where the BACE hydrolysis site is between the leucine (L) and aspartic acid (D). This APP variant contains asparagine (N) and leucine (L) point mutations at the P₂ and P₁ position respectively. When compared to the analogous wild type APP sequence (EVKMDAEIF), which has lysine (K) and methionine (M) at the P₂ and P₁ position, the mutations in the Swedish sequence account for a more than 60-fold increase in the $K_{cat}/K_{M}$.

Additionally, people with the Swedish mutation are more likely to develop early onset Alzheimer’s Disease due to the enhanced rate of β-cleavage and subsequent...

Figure 4: Prochelator strategy for sequestering copper in AD
Aβ production. Other purportedly superior substrates have been identified in the literature, and some were evaluated as BACE substrates via an LC-MS assay (Table 1), but SWH was determined to be the best sequence tested at the time of screening.

**Table 1: BACE substrates evaluated during the development of SWH**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Name</th>
<th>Product Formation after 10h Incubation with BACE</th>
</tr>
</thead>
<tbody>
<tr>
<td>EVNL-DAEF</td>
<td>SW</td>
<td>50%</td>
</tr>
<tr>
<td>EVNL-DAHF</td>
<td>SWH</td>
<td>50%</td>
</tr>
<tr>
<td>EIDL-MVLD</td>
<td>Peptide OK95</td>
<td>20%</td>
</tr>
<tr>
<td>EIDL-SSH</td>
<td>OK-SSH</td>
<td>5%</td>
</tr>
<tr>
<td>EVNL-SSH</td>
<td>SW-SSH</td>
<td>3.5%</td>
</tr>
<tr>
<td>DETL-DAHF</td>
<td>Proteomics 1103</td>
<td>1%</td>
</tr>
<tr>
<td>APSL-DAHF</td>
<td>Proteomics 2103</td>
<td>0.5%</td>
</tr>
<tr>
<td>ASNL-DAHF</td>
<td>Proteomics 3103</td>
<td>Not Detected</td>
</tr>
<tr>
<td>EI(Thi)(Thi)-(Nva)AHF</td>
<td>Kyoto104</td>
<td>25%</td>
</tr>
<tr>
<td>EVNF-EVEF</td>
<td>Merck105</td>
<td>85%</td>
</tr>
</tbody>
</table>

Amino acid single letter abbreviations listed with a (-) representing the putative BACE cleavage site. SW sequence is Swedish mutant APP while OK-SSH and SW-SSH incorporate the first four amino acids from Peptide OK and SW with an SSH ATCUN motif.

The Swedish mutant sequence was modified to incorporate a histidine (H) in the \( P_3' \) position, replacing the native glutamic acid (E). We therefore nicknamed our prochelator SWH representing the Swedish mutant APP sequence with a histidine substitution. Additionally, a tryptophan-containing tag (WADR) was incorporated into the peptide sequence to facilitate concentration determination by taking advantage of the absorptivity of tryptophan’s indole side chain (5625 M\(^{-1}\)cm\(^{-1}\) \( \lambda_{\text{max}} = 280 \) nm). The tag also contains an arginine (R) residue, which has a guanidinium side chain that is positively charged at neutral pH and aids solubility in aqueous solution. The tryptophan
tag does not significantly interfere with enzyme recognition or proteolysis because it is positioned outside the active site as shown in the crystal structure of BACE complexed with a peptide inhibitor.\textsuperscript{100}

After cleavage by BACE, the resulting chelator peptide, termed CP, has the sequence H$_2$N-DAHFWADR that has a known copper-binding site called an ATCUN (amino terminal Cu and Ni binding) motif found in human serum albumin and other copper chaperone proteins.\textsuperscript{101} The ATCUN site is created by the free amino terminus, two deprotonated amide nitrogens from the aspartic acid and alanine residues, and the imidizole side chain of histidine. These four nitrogen donor atoms create a very stable, uncharged, square planar complex when bound to Cu(II). This same motif has also been shown to prevent copper-induced reactive oxygen species.\textsuperscript{102} These features are critical because we want to not only sequester copper from A\textbeta, but also prevent generation of hydroxyl radicals via stabilization of the Cu(II) ion.

2.2.2 Enzyme Reactivity: Prochelator-to-chelator conversion by BACE

2.2.2.1 LC-MS Assay

An LC-MS method was first used to investigate the conversion of prochelator to chelator by enzymatic cleavage. This method utilizes UV detection to monitor a decrease in the prochelator peak area with a corresponding increase in the chelator peak area over time. The masses for the starting peptides and cleavage products were validated by in-line mass spectrometry, verifying that BACE cleavage occurred at the expected location. Figure 5 shows a sample LC-MS chromatogram in which SWH had been incubated with BACE for more than 24 hours. The prochelator substrate peak has been largely digested but is still present. The substrate and both cleavage products are easily detectable in the total ion chromatogram but only SWH and CP are visible in the UV chromatogram due to the tryptophan residue in both peptides.
155 μM SWH dissolved in 0.1 M sodium acetate buffer pH 4.5. Reaction initiated by addition of 10 μL purified BACE (0.6 mg/mL). 16 μL reaction aliquots were diluted in 8M urea to quench reaction at specific timepoints. Displayed from top to bottom are the total ion chromatogram, UV chromatogram at 280 nm, and extracted ion chromatograms for SWH, CP, and N-terminal cleavage product respectively. The expected M+H value in the extraction ion chromatograms refers to the mass of the peptide plus a proton.
Peak detection at 280 nm allows for a direct comparison of the prochelator and chelator peak areas. The only amino acids that significantly absorb light at this wavelength are tryptophan and tyrosine. The intact prochelator, SWH, and the C-terminal cleavage product, CP, each contain a single tryptophan, and thus have similar extinction coefficients at 280 nm. Likewise, the SW control peptide (EVNLDAEFWHDR) and its C-terminal cleavage product (DAEFWHDR) also contain a single tryptophan.

The percent product formed from each substrate was calculated by using Equation 1.

\[
\text{Eq. 1: } \% \text{ Product} = \frac{\text{Peak Area of Product}}{\text{Peak Area of Product} + \text{Peak Area of Substrate}}
\]

Figure 6 shows the percent product of each peptide formed as a function of time when incubated with BACE. The similar rates of hydrolysis confirm our expectation that the mutation of a single amino acid residue (namely histidine for glutamic acid in the P_3' position) would not greatly alter the ability of the SWH prochelator to be cleaved by BACE.
155 µM SWH (prochelator) or SW (native Swedish mutant APP sequence) dissolved in 0.1 M sodium acetate buffer pH 4.5. Reaction initiated by addition of 10 µL purified BACE (0.6 mg/mL). 16 µL reaction aliquots were diluted in 8 M urea to quench reaction at specified time points. Quenched reactions were analyzed via LC-MS and % product determined by the equation above. Rate of product formation determined by slope of the linear fit.

Multiple other BACE substrate peptides identified in the literature were compared to SWH in an effort to ascertain the best possible substrate for the enzyme. Jordan Tang’s group at the University of Oklahoma first employed a combinatorial approach to evaluate the amino acid preference of BACE at each of the P₄ to P₄’ sites. From this, they identified “Peptide OK” which is reported to possess Kcat/KM properties 8-times higher than that of the Swedish mutant APP sequence. The catalytic rates in this publication were determined by directly ratioing the peak intensity of the substrate and product mass peaks as observed via MALDI-TOF. This method is similar to the one
described above, but with several critical differences. In MALDI-TOF experiments, the peak intensity of a particular analyte is dependent on multiple factors in addition to concentration. Peptides of different lengths, particularly if they contain several charged residues, as Peptide OK does, will have different proton affinities as well as different ionization potentials. This means that when two peptides of different lengths are ionized via laser desorption they will not only ionize differently out of the matrix, but also “fly” differently in the time-of-flight analyzer because of their varying proton affinities. Therefore, a sample containing two peptides in equal concentration is unlikely to result in equal peak intensities for those two peptides in the MALDI-TOF spectrum. This discrepancy is exemplified by the R² for the calibration curve reported in this publication of 0.92. The HPLC procedure described above routinely yields calibration curves with correlation coefficients >0.99. For these reasons, Peptide OK was viewed as an unvalidated BACE substrate.

In 2009, Dennis Selkoe and coworkers used quantitative proteomics to identify 68 putative BACE substrates. From these substrates, we selected three sequences which contain an aspartic acid-alanine motif in the P₁' and P₂' positions respectively. The D-A sequence not only mimics the Swedish mutant APP, but also the N-terminus of human serum albumin, whose ATCUN motif (DAH) is known to prevent copper from redox cycling.

The last substrate considered during selectively screening came from a group in Kyoto Japan in 2011 who identified a BACE substrate sequence that incorporated the unnatural amino acids thienyl-thienyl-norvaline in the P₂ to the P₁' sites. This sequence was reported to be hydrolyzed by BACE up to 10-times faster than the Swedish mutant sequence when assayed via LC-MS.
While not part of our initial screen, in 2012 a BACE substrate sequence used by Merck to screen BACE inhibitors was evaluated using our LC-MS method. It was determined that this sequence (EVNFEVEF) was the best substrate tested to date. Unfortunately, as we were unaware of this substrate during the development of SWH, it was not incorporated into our prochelator sequence. Modification of the Merck substrate into a BACE-activated prochelator is planned for future experiments and is discussed in further detail in Chapter 3.

The putative BACE substrates described above were synthesized, purified, and evaluated for their ability to be efficiently cleaved by BACE via the LC-MS. As can be seen from Table 1, the SWH sequence was identified as the best BACE substrate at the time, and subsequently was used as the prochelator sequence for future experiments.
2.2.2.2 Calcein Assay

To confirm that SWH is hydrolyzed by BACE into a product that binds copper, a competition assay with the fluorescent molecule calcein (Figure 7) was employed.

![Calcein molecule](image)

**Figure 7: Calcein**

Adding 1.5 equivalents of Cu(II) to a calcein solution at pH 7.4 significantly quenches calcein’s fluorescence upon chelation. Titration of a quenched calcein solution with CP restores the fluorescence in a dose dependent manner, indicating that CP stoichiometrically displaces copper from calcein. The assay is represented schematically in Figure 8.

![Calcein Assay](image)

**Figure 8: Calcein Assay**

By using a copper-quenched calcein solution as an indicator, aliquots of the SWH + BACE reaction mixture were analyzed to determine the presence of activated chelator by way of an increased calcein fluorescence signal. The amount of product generated by the enzymatic reaction was first determined by HPLC after 24 hours. Quantitation of the product peak areas allowed for the determination of appropriately-sized aliquots to
be added to the calcein indicator solution. Figure 9 shows that when CP is added directly to the quenched calcein solution (burgundy triangles), a dose-dependent increase in the fluorescence signal is observed. Conversely, the prochelator, SWH, does not restore the fluorescence signal (green squares). When SWH is incubated with BACE and converted to CP, the fluorescence signal increases (red circles) similarly to the CP controls, indicating the product formed from enzymatic hydrolysis is in fact the desired copper chelator. The control peptide, SW, is also a BACE substrate but the hydrolysis products are not copper binding molecules and thus do not induce a fluorescence increase (blue diamonds)

**Figure 9**: CP production monitored via calcein fluorescence

Calcein (1 µM) and CuSO₄ (1.5 µM) dissolved in 3 mL 50 mM HEPES pH 7.4 buffer in a fluorescence cuvette. 0.25 µM (~10 µL) CP, SWH, or reaction mixtures with BACE were added and allowed to equilibrate for 5 min. Increasing fluorescence signal indicates displacement of copper from calcein. λ<sub>ex/em</sub> = 485/515 nm.
2.2.3 Serum Stability and D-amino acid variants

Biological fluid stability is vital for drug candidate compounds in order to achieve proper pharmacological effects. Compounds with poor stability are rapidly decomposed and excreted from the body, and thus have poor activity in vivo.\textsuperscript{106} It is therefore important to quantifiably determine the stability of drug candidate compounds in early research stages, such that unstable candidates can be discarded or structurally altered in an attempt to improve stability. Biological fluids contain numerous hydrolytic enzymes, such as proteases and peptidases, potentially capable of degrading or destroying drug candidate compounds. Thus, a number of structural modification strategies for improving the stability of a test compound in biological fluid have been characterized, such as using D-amino acids, replacing functional groups or introducing steric hindrance.\textsuperscript{106}

The two most commonly tested biological fluids are blood plasma and blood serum. Plasma is obtained by removing blood cells from a whole blood sample treated with anticoagulant. Serum is obtained by removing the coagulated blood cells and platelets from a clotted blood sample. The two fluids differ only in their content of certain clotting factors (fibrinogens), and the presence of anticoagulants. Because no anticoagulant is present in serum, some additional protease activity is retained that is inhibited by anticoagulants in plasma.\textsuperscript{106} For this reason, we chose serum as the media in which to evaluate SWH stability.

The half-life of SWH and D-SWH (all D-amino acid variant) in blood serum was analyzed by an LC-MS stability assay adapted from the literature.\textsuperscript{107} Caffeine was used as an internal standard. The results, shown chromatographically in Figure 10,

\textsuperscript{2} Work performed primarily by undergraduate student Marcus Kaplan and reprinted in part with permission from his end-of-year reports.
demonstrate that SWH is degraded in blood serum, whereas D-SWH is stable across the time points tested.

Figure 10: LC Chromatograms of SWH and D-SWH

100 µM SWH and D-SWH incubated with male type AB blood serum at 37 °C for 300 min. At time points specified 100 µL reaction mixture was removed and quenched with cold acetonitrile. 75 µM caffeine added as an internal standard and samples analyzed via LC-MS. SWH is degraded in serum as evidenced by a decreasing peak area whereas the D-SWH peptide is stable.

By plotting the natural log of the ratio of peptide to caffeine peak areas, the half-life of SWH can be determined according to Equation 2. Figure 11 demonstrates a good linear fit of the chromatographic data with a slope of -0.0097 corresponding to a half-life for SWH in blood serum of about 71 min.

Equation 2: Half-life = ln(0.5)/slope of regression line
SWH has a short, but biologically relevant half-life in blood serum. D-SWH, however, appears much more stable and would make a better drug candidate if able to be recognized and hydrolyzed by BACE. Unfortunately D-SWH, as well as any other variants containing D-amino acid substitutions in the BACE recognition site (EVNLDAHF), rendered the peptide resistant to BACE hydrolysis.

Table 2 summarizes our attempts to incorporate D-amino acids into the SWH sequence and still yield a suitable BACE substrate. Unfortunately, none of the D-amino acid variants tested were converted to more than 30% product after 24-hour BACE incubation. The peptide with the highest conversion rate, 8L, only contained D-amino acids in the tag region outside of the enzyme recognition site. Therefore, only the native, all L-SWH peptide was carried forward to subsequent experiments.
Table 2: D-SWH variants evaluated as BACE substrates

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
<th>% Product after 24 h w/ BACE</th>
</tr>
</thead>
<tbody>
<tr>
<td>E(D) V(D) N(D) L(D) - D(D) A(D) H(D) F(D) W(D)</td>
<td>D-SWH</td>
<td>Not Detected</td>
</tr>
<tr>
<td>E(D) V(D) N(D) L-D A(D) H(D) F(D) W(D) A(D)</td>
<td>2L</td>
<td>Not Detected</td>
</tr>
<tr>
<td>E(D) V(D) N L-D A H(D) F(D) W(D) A(D) D(D)</td>
<td>4L</td>
<td>Not Detected</td>
</tr>
<tr>
<td>E V N L-D A H F W A A D R</td>
<td>SWH</td>
<td>50%</td>
</tr>
</tbody>
</table>

2.2.4 SWH and CP copper binding

2.2.4.1 CP copper binding

The conditional stability constant (log K') for the binding of CP to Cu(II) at pH 7.4 was determined spectrophotometrically through a competition study with nitrilotriacetic acid (NTA) and corrected for the Cu(NTA)(HEPES) ternary complex as described in Section 2.4.6. A sample titration is presented in Figure 12. Copper bound by the ATCUN motif in CP exhibits a d-d transition band at 526 nm with an extinction coefficient of 110 M⁻¹ cm⁻¹. Upon titration of the competitive chelator NTA, the copper is displaced from CP resulting in a decrease in the CP(Cu) spectral signal and a corresponding increase in the NTA(Cu) complex spectral signal. From this titration a log K' = 12.6 ± 0.1 was determined for CP binding to Cu(II) at pH 7.4. This value is in agreement with the N-terminal binding site of human serum albumin, which has the same copper binding amino acid sequence.¹⁰⁹
Figure 12: Titration of CP(Cu) with NTA

1 mL of CP(Cu) (1 mM) prepared in a 1 cm cuvette in 50 mM HEPES buffer pH 7.4. 1-2 µL aliquots of NTA (1 M) added, mixed, and allowed to equilibrate for 5 min. Spectrum recorded on UV-Vis spectrophotometer at RT. Decrease in CP(Cu) signal and corresponding increase in NTA(Cu) signal indicate copper transfer from CP to NTA.

2.2.4.2 SWH copper binding

The log $K'$ of SWH was determined qualitatively at pH 7.4 with a fluorescence quenching assay. Tryptophan has inherent fluorescence properties when excited at 280 nm. By monitoring the copper-induced fluorescence quenching of tryptophan we can determine whether copper is bound to the SWH prochelator. Figure 13 shows that when excited at 280 nm, tryptophan has significant fluorescence at 361 nm. Addition of an equimolar amount of copper to the solution induces a roughly 50% quenching in the tryptophan fluorescence signal. This is most likely due to copper’s interaction with the
lone histidine residue in SWH. The fluorescence is quickly restored when one equivalent of NTA is added to the solution because NTA displaces the copper from SWH. When a weaker competitive chelator, glycylglycine is added to a quenched solution, the fluorescence again quickly returns, indicating that glycylglycine is also able to displace copper from SWH.

**Figure 13: SWH copper binding study**

SWH (10 µM) dissolved in 3 mL 50 mM HEPES buffer pH 7.4 in a fluorescence cuvette. 1 eq CuSO₄ added and allowed to equilibrate for 5 min. Addition of 1 eq NTA or glycylglycine restores fluorescence indicating copper transfer from SWH to the competitive chelator. λₑₓ/ₑ𝐦 = 280/361 nm.
These results indicate that even though the prochelator SWH is able to bind copper, it is thermodynamically weaker than glycylglycine at pH 7.4. With a log $K'$ of less than 4.7 (that of glycylglycine), SWH would be unable to strip copper from Aβ or any other biologically relevant compound. Conversion of SWH to CP by BACE affords a copper chelator with a log $K'$ of 12.6 at pH 7.4. This suggests the chelator is thermodynamically strong enough to displace copper from Aβ, which has a log $K' = 9.4^{110}$. Results from the copper chelation studies are summarized in Table 3.

**Table 3: Stability constants of selected copper chelators**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Sequence</th>
<th>Log $K'$ at pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlyGly</td>
<td>Glycylglycine</td>
<td>GG</td>
<td>4.7$^{111}$</td>
</tr>
<tr>
<td>SWH</td>
<td>Prochelator</td>
<td>EVNLDAHFWADR</td>
<td>&lt; 4.7</td>
</tr>
<tr>
<td>CP</td>
<td>Chelator</td>
<td>H$_2$N-DAHFWADR</td>
<td>12.6 ± 0.1</td>
</tr>
<tr>
<td>Aβ</td>
<td>Aβ(1-40)</td>
<td>See 2.4.3 Preparation of Aβ</td>
<td>9.4$^{110}$</td>
</tr>
<tr>
<td>HSA</td>
<td>albumin</td>
<td>N-term = H$_2$N-DAHK...</td>
<td>12.0$^{109}$</td>
</tr>
</tbody>
</table>

**2.2.5 Copper Transfer from Aβ to CP**

Even though CP is a thermodynamically stronger copper chelator than Aβ, if the Aβ(Cu) complex is kinetically inert, CP would still be unable to sequester the metal. In order to test this hypothesis, the fluorescence of the tyrosine in Aβ and the tryptophan tag in CP was utilized to monitor the direct transfer of Cu(II) from Aβ to CP. The binding of paramagnetic metal ions to peptides is known to quench the fluorescence of the indole side chain of tryptophan and the phenol side chain of tyrosine. As expected, the addition of copper to SWH and CP independently quenches the fluorescence of each...
compound. As can be seen from the inset in Figure 14, the addition of one equivalent of Cu(II) to an Aβ stock solution at pH 7.4 quenches the fluorescence of the lone tyrosine residue in Aβ confirming metal binding. Subsequent addition of CP to the Aβ(Cu) solution (green squares) results in a consistent quenching of the tryptophan fluorescence signal of CP until 1 equivalent of CP is reached, after which the tryptophan fluorescence increases linearly with concentration. An expected increase in Aβ’s tyrosine fluorescence upon removal of Cu²⁺ is not observed due to the much broader and more intense tryptophan signal. Quenching of the tryptophan in CP implies that the chelator is stoichiometrically stripping copper from Aβ and consequently, that the copper in the Aβ(Cu) complex is kinetically labile. The experiment was repeated using the SWH prochelator (blue circles), which showed no fluorescence quenching, indicating the prochelator was unable to strip Cu from Aβ. Sequestration of copper form Aβ by CP is consistent with the thermodynamic data in Table 3.
Figure 14: Copper transfer from $\text{A}$β to CP

$\text{A}$β (10 $\mu$M) diluted in 3 mL 50 mM HEPES buffer pH 7.4 in a fluorescence cuvette. CuSO$_4$ (10 $\mu$M) added to quench the tyrosine residue in $\text{A}$β, $\lambda_{\text{ex}} = 280$ nm (inset). CP and SWH aliquots (2 $\mu$M) added and allowed to equilibrate for 5 min. Quenching of CP fluorescence signal indicates copper transfer from $\text{A}$β to CP. SWH is not quenched indicating lack of copper transfer. $\lambda_{\text{ex/em}} = 280/361$ nm.
2.2.6 Protection against hydroxyl radical production: Deoxyribose Assay

In the presence of a biologically relevant reducing agent such as ascorbate, copper bound to Aβ is able to cycle between the cuprous and cupric form reducing hydrogen peroxide and generating hydroxyl radicals via the Fenton reaction shown in Figure 15.\(^85\)

![Figure 15: Fenton Reaction with copper](image)

Hydroxyl radicals are the most reactive of the reactive oxygen species associated with oxidative stress with a half-life on the order of \(10^{-9}\) sec. As such, hydroxyl radicals will react with biomolecules within a few angstroms of where they are generated.\(^{112}\) Hydroxyl radicals are impossible to observe directly in vivo, but evidence of their existence is widespread in the AD brain in the form of lipid, DNA, RNA, and protein oxidation products.\(^{113}\)

The ability of CP to protect against hydroxyl radical production is an important characteristic for a therapeutic directed against AD. The deoxyribose assay was used to quantitate the amount of hydroxyl radicals produced when copper is bound to SWH and CP. In this assay, a mixture of copper, ascorbic acid and hydrogen peroxide
generate hydroxyl radicals through Fenton-like chemistry. Hydroxyl radicals attack 2-deoxyribose to form malondialdehyde, which upon heating with thiobarbituric acid produce a pink chromophore. Chelators that prevent copper from redox cycling in the presence of ascorbic acid and hydrogen peroxide result in less chromophore formation and is observed as a decreased Abs at 490 nm.

**Figure 16: Protective effects of CP and SWH as measured by the deoxyribose assay**

Effect of CP and SWH on deoxyribose degradation by OH\(^+\) generated by Cu-promoted Fenton chemistry. Conditions: 0-100 µM SWH and CP, 100 µM H\(_2\)O\(_2\), 10 µM Cu(SO\(_4\))\(_2\), 2 mM ascorbic acid, and 15 mM 2-deoxyribose in 50 mM NaH\(_2\)PO\(_4\) buffer pH 7.4. A decrease in A/A\(_0\) indicates a protective effect.

The absorbance data displayed in Figure 16 is normalized to zero chelator where maximum production of hydroxyl radicals is designated as 1. CP efficiently protects against radical formation by chelating copper and preventing up to 80% of Fenton-like chemistry at a 1:1 CP:Cu ratio (10 µM). This protective effect is consistent with other
reports in the literature on similar peptide sequences.\textsuperscript{101} SWH demonstrates some protective effects, but this result is most likely due to radical quenching, particularly at high concentrations of prochelator.

The cleavage product resulting from Kyoto (Table 1) incubation with BACE was also analyzed with the deoxyribose assay. This peptide has the sequence H\textsubscript{2}N-Nva-A-H-F-R-W with the only significant difference from CP being the aspartic acid to norvaline substitution in the \( P_1 \)' position. The results were similar to that of CP suggesting the protective properties from hydroxyl radical production are a more general characteristic of ATCUN motifs as opposed to specific motifs being more protective than others.

\textbf{2.2.7 Protection against hydrogen peroxide production: Amplex Red Assay}

Under reducing conditions the A\( \beta \)(Cu) complex has been shown to react with \( \text{O}_2 \) to produce hydrogen peroxide.\textsuperscript{114} While hydrogen peroxide itself is not toxic, its formation can be potentially dangerous if produced in the presence of a redox-active metal as it is a key precursor to the formation of hydroxyl radicals via the Fenton reaction. An Amplex Red assay was used to measure the ability of the chelator and prochelator to protect against \( \text{H}_2\text{O}_2 \) production. In this assay, Amplex Red (10-acetyl-3,7-dihydrophenoxazine) reacts with \( \text{H}_2\text{O}_2 \) in a 1:1 stoichiometry in the presence of horseradish peroxidase to produce a red-fluorescent oxidation product, resorufin. Comparison to a standard curve allows the amount of \( \text{H}_2\text{O}_2 \) formed in solution to be accurately quantitated. The results shown in Figure 17 demonstrate that unbound copper, as well as copper ligated by A\( \beta \) or SWH, in the presence of ascorbic acid (AA), generate approximately equal amounts of hydrogen peroxide. However, CP is able to decrease the amount of peroxide produced in a dose dependent manner up to \( \sim 50\% \) at
2:1 CP:Cu ratio. This result, in combination with the deoxyribose data presented above, support the hypothesis that copper bound to CP greatly diminishes the metal’s ability to catalyze oxidative stress reactions as compared to ligation by Aβ.

Figure 17: Amplex red assay measuring hydrogen peroxide production

H$_2$O$_2$ production from Aβ(Cu) (200 nM) in the presence of ascorbic acid (AA) (10 µM), SWH (200 nM) and CP (50-400 nM). Aβ-negative control is also shown.

2.2.8 CP inhibits and reverses Cu-induced Aβ aggregation

Metal ions, particularly copper and zinc are known to exacerbate Aβ aggregation. These aggregates eventually form more ordered fibrils, protofibrils, and eventually the plaques characteristic of AD. By sequestering Cu(II) from Aβ, CP
displays an ability to inhibit Cu(II)-induced Aβ aggregate formation, as verified by the light-scattering turbidity assay shown in Figure 18. As expected, SWH is unable to inhibit aggregate formation while CP shows a protective effect at 1:1 CP:Cu stoichiometry. This result is consistent with data from peptides of similar sequences reported by others. Predictably, CP is not as effective at binding Zn(II) and preventing Zn(II)-induced aggregation. Importantly, the reaction mixture from SWH and BACE incubations (but not SW + BACE) is also able to prevent aggregate formation. Furthermore, the SWH + BACE condition was also shown to disaggregate pre-formed Aβ(Cu) aggregates, as shown by the green bars. This experiment provides a full in vitro demonstration of the hypothesis shown in Figure 4. The prochelator, SWH, is converted to the chelator, CP, by BACE hydrolysis. CP then sequesters copper from Aβ aggregates and induces dissagregation of peptide monomers.

![Figure 18: Aβ aggregation assay](image)

Turbidity of 10 μM Aβ samples in HEPES buffer pH 7.4, as determined by the normalized change in A$_{405nm}$. Where indicated, 1 equiv of Cu(Gly)$_2$, ZnCl$_2$, CP, or SWH were added and incubated at 37 °C for 1 h to monitor aggregation prevention (blue bars). Products from SW or SWH plus BACE reactions were also tested for disaggregation of preformed Aβ(Cu) aggregates (green dotted bars).
2.3 Conclusions

In summary, we have developed a prochelator peptide SWH that is efficiently hydrolyzed by \(\beta\)-Secretase to reveal a high affinity copper chelator CP. CP was shown to be able to sequester copper from A\(\beta\), protect against oxidative stress, and reverse copper-induced A\(\beta\) aggregate formation. These characteristics make SWH a strong drug candidate in vitro and warrant further investigation in living systems. Preliminary studies into the plasma stability of SWH revealed that incorporation of D-amino acids increase plasma stability, but that substitutions around the enzyme recognition site result in the peptide no longer being a suitable BACE substrate. This observation, in addition to data from the multiple other BACE substrates evaluated, suggest that BACE is fairly specific in its substrate preference. However, SWH does not seem to have similar specificity for BACE as it is degraded relatively quickly in serum. Future studies should be cognizant of potential stability and specificity issues arising from non-BACE mediated hydrolysis.

2.4 Experimental

2.4.1 Materials and Instrumentation

All chemicals and solvents were obtained from Sigma-Aldrich and used without further purification unless otherwise noted. All water was nanopure. Peptides were synthesized on a Protein Technologies PS3 automated peptide synthesizer and purified on a Waters 600 HPLC. Liquid chromatography-electrospray mass spectrometry (LC-MS) data were collected on an Agilent 1100 Series HPLC in line with a LC/MSD trap and a Daly conversion dynode detector. UV-Vis spectra were recorded on a Cary 50 UV-Vis spectrophotometer. Turbidity, deoxyribse, and Amplex Red assays were conducted
on a Perkin Elmer Victor 1420 plate reader. Fluorescence data were recorded on a FluoroLog-3 fluorimeter from HORIBA Jobin Yvon.

2.4.2 Preparation of Peptides

Peptides were synthesized in 0.1 mmol scale on PAL-PEG-PS resin (Applied Biosystems). Standard Fmoc (9-fluorenylmethoxy-carbonyl)-protected amino acids (Chem-Impex and Novabiochem) were coupled in 20 min cycles with HBTU (O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate) (Novabiochem) and N-methylmorpholine (NMM) (Acros) in N,N'-dimethylformamide (DMF) (Caledon). Fmoc protecting groups were removed by using 20% piperidine in DMF. The N-termini of peptides were acetylated using acetic anhydride and NMM. Cleavage from the resin and removal of side chain protecting groups was accomplished by treating resin with a 10-mL mixture of 95% trifluoroacetic acid (TFA) and 2.5% triisopropylsilane (TIS) under nitrogen while shaking for 4 h. Peptide was precipitated from solution by evaporating off TFA with a nitrogen stream, followed by three washes with diethyl ether (Caledon). Purification was accomplished by semi-preparative reversed-phase HPLC on a YMC C18 column with a linear 40 min gradient from 7 to 70% acetonitrile in water with 0.1% TFA. Purity was validated to be greater than 95% by analytical HPLC. Mass of each peptide was determined by ESI-MS.

2.4.3 Preparation of Aβ

The Aβ1-42 peptide was purchased from either Genscript or EZBioLab and validated by the supplier to be > 95% pure (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVIA). A portion (1 mg) of Aβ was dissolved in 600 µL of 1% ammonium hydroxide in de-ionized water by sonicating 1 min on, 30 s off, and 1 min on. The solution was filtered through a syringe filter (GE Water, Nylon 0.22 µM, 3 mm). A second aliquot of 400 µL 1% ammonium
hydroxide was used to rinse the vial and filter of any residual peptide. The concentration of this stock solution was determined by the Pierce MicroBCA assay and validated by measuring the tyrosinate absorption (295 nm, $\varepsilon = 2480 \text{ M}^{-1}\text{cm}^{-1}$) at pH 12. The stock solution was portioned into aliquots and stored at -20 °C and used within 5 days of preparation.

### 2.4.4 LC-MS Kinetic Assay of BACE Activity

#### 2.4.4.1 LC-MS Assay

SWH prochelator and Swedish mutant APP control peptide (SW) were dissolved in 10% glacial acetic acid and the pH was adjusted to 4.5 with 100 mM NaOH. Water was added to achieve final concentrations of 155 µM. Aliquots of 350 µL were pipetted into eppendorf tubes and 10 µL β-secretase (C-terminal FLAG-tagged, extracellular domain; Sigma-Aldrich) (0.6 mg/mL) was added to initiate the reaction. Samples were allowed to vortex slowly at 25 °C. Aliquots of 16 µL were removed from the reaction mixture every 5–30 min and diluted into 25 µL of 8 M urea in HPLC vials to quench the reaction. Samples were analyzed on a Varian Polaris C18 reverse phase column (150 mm x 1 mm) with a linear 20-min gradient from 3–55% acetonitrile in water with 0.3% formic acid. Signal was detected at 280 nm on a UV detector.

#### 2.4.4.2 Calcein Assay

Calcein assay was performed by dissolving calcein in sodium bicarbonate and diluting to 1 µM in 3 mL 50 mM HEPES pH 7.4. 1.5 µM CuSO$_4$ was added from a concentrated stock solution and allowed to equilibrate for 5 min. Solutions were prepared in a 3 mL fluorescence cuvette. The excitation and emission wavelengths for calcein are 487 nm and 515 nm respectively. Figure 19 demonstrates the fluorescence quenching effect copper has when added to a calcein stock solution at pH 7.4.
Calcein (1 µM) dissolved in 50 mM HEPES buffer pH 7.4 and titrated with CuSO₄ (0.25 µM) aliquots. Calcein fluorescence decreases with increasing copper concentration due to quenching effects. \( \lambda_{ex/em} = 487/515 \text{ nm} \)

Enzyme reactions were performed as described above. After 24 hours, the enzyme reaction mixture was analyzed via HPLC and the percent product was calculated as described. The percent product was used to calculate the concentration of CP in the reaction mixture and subsequently the appropriate aliquot volume (~ 10 µL) to deliver 250 nM CP (0.25 eq) to the quenched calcein solution. After aliquot addition, the solution was mixed via aspiration with a pipette and allowed to equilibrate in the cuvette for 5 min.

### 2.4.5 Serum Stability Assays

SWH (all L-amino acids) and D-SWH (all D-amino acids) were synthesized for the serum stability assay. These test peptides were screened for stability in human male type AB blood serum by adapting a procedure described by Marastoni, et al. Serum samples were prepared as described in Table 4.
The serum was stored in a freezer and was incubated for 3 hours at 37 °C to activate enzymes prior to sample preparation. After incubation, the serum was centrifuged (3000 rpm for 10 minutes) and decanted to remove any particulate matter. Samples were diluted to a total volume of 1 mL by adding 100 µL of 1 mM peptide stock in water to 900 µL of decanted serum to a final peptide concentration of 100 µM. A serum blank was prepared by adding 100 µL of water to 900 µL of serum. Each reaction mixture was vortexed and incubated at 37 °C for 5 hours.

At each time point, a 100 µL aliquot was removed from each reaction mixture and mixed with 200 µL of cold acetonitrile to quench. The aliquots were vortexed and centrifuged at 3000 rpm for 5 minutes. 75 µM caffeine in water was added as an internal standard to each sample and analyzed via LC-MS.

### 2.4.6 Copper Binding

#### 2.4.6.1 CP Copper Binding

Cu(II) sulfate solution (100 mM) was prepared from solid Cu(SO$_4$)$_2$·5H$_2$O and standardized with 0.0500 M EDTA to a murexide endpoint in ammonia buffer. Lyophilized CP was dissolved in H$_2$O and the concentration was determined spectrophotometrically by pipetting small aliquots (3-5 µL) into 1 mL 8 M urea and recording the absorbance at 280 nm. The extinction coefficient of tryptophan under these conditions is 5635 M$^-$$^1$cm$^-$$^1$. The extinction coefficient for the d-d transition band of

<table>
<thead>
<tr>
<th>Assay Parameters</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Peptide Concentration</td>
<td>100 µM</td>
</tr>
<tr>
<td>Serum pre-incubation</td>
<td>180 m at 37 °C</td>
</tr>
<tr>
<td>Sample incubation</td>
<td>0-300 m at 37 °C</td>
</tr>
<tr>
<td>Reaction Quenching</td>
<td>200 µL cold acetonitrile</td>
</tr>
</tbody>
</table>
CP(Cu) was determined by titration of Cu$^{2+}$ into 1 mM CP in 50 mM HEPES (N-(2-hydroxyethyl)-piperazine-N’-2-ethansulfonic acid) buffer pH 7.4. The complex shows an absorption maximum at 526 nm with a molar absorptivity of 110 M$^{-1}$cm$^{-1}$ (Figure 20).

![Figure 20: CP(Cu) Extinction Coefficient](image)

CP (1 mM) titrated with 0.1 Eq CuSO$_4$ aliquots. Absorbance peak at 526 nm grows linearly with copper addition. Linear fitting through 0 provides an extinction coefficient of 110 M$^{-1}$cm$^{-1}$ for the d-d transition band of the CP(Cu) complex.

Competition studies were performed by preparing 1 mL of 1 mM CP(Cu) in 50 mM HEPES buffer. The reaction vessel was a 3 mL cuvette and all titrations were carried out at 25 °C. Aliquots (1-2 µL) of the competitive chelator NTA (1 M) were pipetted into CP(Cu) solutions and monitored spectrophotometrically. After each addition, solutions were manually mixed and equilibrated for 5 min before data were collected. Conditional stability constants were calculated on Microsoft Excel using the formulas listed below. The conditional stability constant (K’) for NTA was calculated to be 10.68 at pH 7.4 from the overall log β of 12.7 for the 1:1 Cu:NTA complex.$^{111}$
\[ [\text{CP}]_{\text{Total}} = [\text{CP}]_{\text{Free}} + [\text{CP(Cu)}] \]

\[ [\text{NTA}]_{\text{Total}} = [\text{NTA}]_{\text{Free}} + [\text{NTA(Cu)}] \]

\[ [\text{Cu}]_{\text{Total}} = [\text{Cu}]_{\text{Free}} + [\text{CP(Cu)}] + [\text{NTA(Cu)}] \]

\[
\text{CP}_{\text{Free}} + \text{Cu}^{2+} \rightarrow \text{CP(Cu)} ; \quad K_{\text{CP(Cu)}}^{\text{app}} = \frac{[\text{CP(Cu)}]}{[\text{Cu}^{2+}][\text{CP}_{\text{Free}}]} 
\]

\[
\text{NTA}_{\text{Free}} + \text{Cu}^{2+} \rightarrow \text{NTA(Cu)} ; \quad K'_{\text{NTA}} = \frac{[\text{NTA(Cu)}]}{[\text{NTA}_{\text{Free}}][\text{Cu}^{2+}]} = 10^{10.68} 
\]

\[
\text{CP(Cu)} + \text{NTA}_{\text{Free}} \Leftrightarrow \text{NTA(Cu)} + \text{CP}_{\text{Free}} ; \quad K_{\text{ex}} = \frac{[\text{CP}_{\text{Free}}][\text{NTA(Cu)}]}{[\text{CP(Cu)}][\text{NTA}_{\text{Free}}]} = \frac{K'_{\text{NTA}}}{K_{\text{CP(Cu)}}} 
\]

\[
K_{\text{CP(Cu)}}^{\text{app}} = \frac{K'_{\text{NTA}}}{K_{\text{ex}}} 
\]

The competition experiment revealed \( K_{\text{CP(Cu)}}^{\text{app}} \) to be \( 10^{12} \). This apparent value can be converted to a conditional constant (\( K' \)) by accounting for the ternary complex that forms between NTA, copper, and HEPES. The conditional stability constant for CP with Cu(II) at pH 7.4 was therefore obtained as \( K' = 10^{12.6} \), according to the following equation, where \( \log K_T \) for the ternary complex was taken to be 0.6.\(^{109}\)

\[
\log(K'_{\text{CP(Cu)}}) = \log K_{\text{CP(Cu)}}^{\text{app}} + \log K_T 
\]

### 2.4.6.2 SWH Copper Binding

The conditional copper binding of the prochelator (SWH) at pH 7.4 was evaluated using a fluorescence-quenching assay. 3 mL of 10 \( \mu \)M SWH in 50 mM HEPES buffer was prepared in a fluorescence cuvette. 1 equiv of CuSO\(_4\) was added to quench the fluorescence signal through copper binding. The excitation and emission wavelengths were 280 nm and 361 nm respectively. 1 equivalent of competitive chelators NTA and Glycylglycine (GlyGly) were added to individual samples of SWH(Cu). Restoration of fluorescence indicated removal of copper from SWH. The
results shown in Figure 13 indicate SWH does not bind copper as strongly as GlyGly thus making its affinity (log $K'$) towards metal ions at pH 7.4 less than 4.7 (calculated from overall log $\beta$ of 5.55).\textsuperscript{111}

**2.4.7 Copper Transfer from Aβ to CP by Fluorescence Quenching**

Stock solutions of Aβ were added to a 3 mL fluorescence cuvette and diluted to 1 mL with HEPES buffer (50 mM HEPES, pH 7.4) to achieve a final concentration of 10 $\mu$M. CuSO$_4$ (10 $\mu$M) was added and a quenching of the fluorescence signal of tyrosine was observed indicating copper binding. Aliquots (0.2 equiv) of CP were titrated into the solution, mixed, and allowed to equilibrate for 5 min. A fluorescence scan was then taken with excitation at 280 nm. Fluorescence at 361 nm was monitored to observe tryptophan signal quenching upon displacement of copper from Aβ to CP (Figure 14). This response is consistent with CP extracting Cu$^{2+}$ from Aβ, which prevents tryptophan emission until the concentration of CP exceeds that of Cu$^{2+}$. The experiment was repeated with the prochelator being titrated into solution (0.2 equiv). Copper-negative controls for all trials were also performed (not shown).

**2.4.8 Assessment of Aβ Aggregation: Turbidity Assay**

All solutions were prepared using Chelex-treated HEPES buffer (50 mM HEPES, 150 mM NaCl, pH 7.4) Metal solutions were prepared fresh daily. Enzymatic cleavage of SWH and SW were performed by incubating 155 $\mu$M SWH or SW in 0.1M sodium acetate buffer pH 4.5 with BACE at room temperature. After 19 h incubation, an aliquot was removed, quenched in 8M urea, and analyzed by LC-MS to determine concentration of cleavage product formation.

Aggregation prevention assays (blue solid bars of Figure 18) were performed by adding reagents to individual wells of a 96-well plate in the following order, with the
final concentrations given in parentheses: HEPES buffer to give a final total volume of 200 µL, Cu(Gly)$_2$ (prepared from CuCl$_2$ with 2 eq. glycine) or ZnCl$_2$ (10 µM), SWH prochelator (10 µM), CP chelator (10 µM), BACE proteolysis products (10 µM), and lastly Aβ (10 µM). Samples were incubated at 37 °C for 2 h with the plate lid on to minimize evaporation. After incubation, the turbidity was measured by light scattering at 405 nm on a plate reader set to collect one reading every min for 4 min with 30 s of shaking in between each reading. Solution turbidity was determined by subtracting the Aβ-negative control from each well and averaging these values over each 4-min period. Error bars represent the standard deviation from a minimum of 3 independent samples.

Dissaggregation assays (green dotted bars of Figure 18) were performed by adding the enzyme proteolysis products (10 µM), to wells of Aβ(Cu) and the corresponding control wells, which had been allowed to aggregate for 2 h. The plate was incubated for an additional 2 h with the lid on to limit evaporation and the degree of light scattering was measured at 405 nm as described above.

### 2.4.9 Amplex Red Assay

Hydrogen peroxide quantitation was performed by following the general assay directions for the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit available from Invitrogen. Reagents were added to individual wells of a 96-well plate in the following order, with final concentrations given in parentheses and a final volume of 50 µL: sodium phosphate buffer (50 mM), CP (50–400 nM), SWH (200 nM), H$_2$O$_2$ (50 nM – 5 µM), Aβ1-42 (200 nM), CuGly$_2$ (200 nM), and ascorbic acid (10 µM). Amplex Red (50 µM; 0.1 U/mL HRP) was added immediately and the plate was incubated at 37 °C for 1 h. The production of the fluorescent resorufin product was measured on a plate reader with λ$_{ex/em}$ = 530/590 nm. The amount of H$_2$O$_2$ produced under each reaction condition was quantified by comparison to a standard curve measured immediately before sample
analysis. (Figure 21) Error bars represent standard deviation from runs done in at least triplicate.

![Graph](image_url)

**Figure 21 H₂O₂ Calibration Curve**

Hydrogen peroxide (0-5 µM) calibration curve as measured by the amplex red assay. λ<sub>ex/em</sub> = 530/590 nm

### 2.4.10 Deoxyribose Assay

The 2-deoxyribose assay was used to measure hydroxyl radical formation. All assays were performed using 50 mM NaH₂PO₄ buffered to pH 7.4. The following reagents were added sequentially to obtain a 100 µL buffered solution with these final concentrations: SWH and CP chelators (3–100 µM), Cu(SO₄) (10 µM), 2-deoxyribose (15 mM), H₂O₂ (100 µM), and ascorbic acid (2 mM). Stock solutions of ascorbic acid and H₂O₂ were prepared fresh daily and reactions were carried out in a polystyrene 96-well cell culture plate. The reaction mixtures were agitated at 37 °C for 1 h, then 100 µL of thiobarbituric acid (TBA) (1% w/v in 50 mM NaOH) and 100 µL of trichloroacetic acid (2.8% w/v in water) were added to quench the reaction. The temperature was increased.
to 100 °C for 20 min, then cooled to room temperature and the absorbance at 490 nm was recorded using a PerkinElmer 1420 plate reader (Figure 22). Values are reported as normalized absorbance ($A / A_0$) where $A_0$ is the absorbance without chelator present and $A$ is the absorbance with chelator added. The value for Cu(SO$_4$)$_2$ alone is $A / A_0 = 1$. Error bars represent standard deviations from measurements done in at least quadruplicate.

**Copper Reduction**

$$\text{Cu}^{2+} + \text{Ascorbate} \rightarrow \text{Cu}^+ + \text{dehydroxyascorbate}$$

**Hydroxyl Radical Formation**

$$\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{OH}^- + \text{OH}^-$$

**Oxidative Damage to 2'-Deoxyribose**

![Chemical reaction diagram](image)

**MDA-TBA Chromophore**

![Chemical reaction diagram](image)

Figure 22: Deoxyribose Assay
3. Converting SWH to CP in Living Cells and the Development of $\beta$-MAP

The experiments described in Chapter 2 support the hypothesis that a $\beta$-secretase (BACE) activated prochelator could alleviate AD-related toxicity in vitro. To explore the utility of SWH in a cellular model, several complicating factors had to be addressed. The SWH and CP peptides must be non-toxic to cells at relevant concentrations. SWH must be specifically hydrolyzed by BACE to form CP and not simply degraded by other cellular enzymes. A suitable cell line and exogenous conditions must be evaluated to replicate copper-mediated toxicity in AD, and finally, once activated, CP must efficiently protect cells from disease conditions. Experiments designed to assess the first two factors: toxicity and BACE-specific hydrolysis of SWH, are addressed in this chapter.

3.1 Background and Significance

The pathological hallmark of an Alzheimer’s Diseased brain is the presence of insoluble neuritic plaques composed primarily of amyloid-beta (A$\beta$).\textsuperscript{115} A$\beta$ is a 37-43 amino acid peptide that is linked to variety of neurotoxic events, including oxidation of lipid membranes, hyperphosphorylation of tau protein, and disruption of calcium homeostasis.\textsuperscript{20,116} Interestingly, the interactions of A$\beta$ with redox active metals like copper, have been shown to exacerbate these effects and greatly enhance the toxicity of A$\beta$.\textsuperscript{117} These molecular events have macrobiological consequences in the form of diseased synapses, neuronal cell death, and eventually the outward symptoms of dementia. Because evidence of A$\beta$ and A$\beta$(Cu) toxicity are widespread in the post-mortem AD brain, preventing A$\beta$ formation, and/or its interaction with metal ions, have been sought-after strategies for potential therapeutic intervention.
Our approach towards mitigating Aβ(Cu) toxicity in cells relies on enzymatic activation of a prochelator to yield a chelator capable of sequestering copper from Aβ, and preventing neurotoxic redox reactions. The success of the enzymatic activation step is critical to the success of our hypothesis because without proper conversion of the prochelator to the active chelator, sequestration of copper and subsequent mitigation of toxicity cannot occur. This activation step is significantly more difficult to achieve and study in cells than in vitro because of the immense increase in the number of enzymes present and possible side reactions that can occur. Fortunately, the enzyme responsible for activation of the prochelator, β-secretase, is involved in Aβ generation and thus has been well studied and documented in the AD literature. We can therefore draw on our knowledge of Aβ production to not only gain insights into how to activate our prochelator by endogenous BACE, but also how to observe and verify activation is taking place.

Production of Aβ results from sequential cleavage of a transmembrane protein called amyloid precursor protein (APP) by two proteases, β- and γ-secretase. β-secretase is a membrane-associated aspartic protease that cleaves APP in the extracellular domain. Hydrolysis creates the N-terminus of Aβ and initiates the amyloidogenic processing of APP. Subsequent cleavage by γ-secretase in the membrane creates the C-terminus and releases the Aβ fragment. An alternative APP processing pathway exists that does not result in neurotoxic Aβ release. Initial hydrolysis by α-secretase, instead of BACE, followed by γ-secretase cleavage releases non-Aβ protein fragments that appear to have some neuroprotective effects, having been shown to enhance memory and learning in mice. Further indicting BACE as a facilitator of AD are observations that genetic mutations that lead either to increased BACE or decreased α-secretase activity result in increased Aβ production and early-onset AD. Conversely, experiments that inhibit
BACE activity including gene knockdown, interfering RNA, or APP mutation, have been shown to reduce amyloid plaque loads and restore cognitive abilities. These results suggest BACE is an abundantly active protein in AD etiology and thus BACE has become a prime target for pharmacological inhibition. We aim to take an inverse approach by taking advantage of BACE activity to activate our prodrug whereby site-directing the chelator to the origin of disease.

To our knowledge, using BACE as the activating agent for a prodrug has not been previously attempted. Therefore, there is no precedent for how to design a BACE targeted prodrug in cells. However, the design features of potent BACE inhibitors, as well as the assays used to gauge their effectiveness, have been extensively studied in both industry and academia resulting in over 400 publications and patents as of early 2012. This literature provides a starting point towards modifying our previously described SWH peptide into a cellular-active prochelator.

Developing potent BACE inhibitors has been challenging due to the broad substrate tolerance and active site of the enzyme. This lack of substrate specificity has been demonstrated, at least in part, by our lab with the range of substrates shown in Chapter 2 to have at least some propensity to be hydrolyzed by BACE. Designing highly potent compounds for enzymes with relatively large and disordered active sites is far more difficult than for those with rigid and well-defined binding pockets. Compounding potency concerns are specificity issues arising from trying to target BACE over other aspartic proteases such as renin, cathepsin D, and BACE 2, which have 29%, 39%, and 79% sequence identities in the active site respectively. Despite these challenges however, there are multiple reports of small molecule BACE inhibitors in the literature with potencies (IC₅₀) ranging from the micromolar down to the nanomolar range that are at least partially selective for BACE. Unfortunately, there do not seem
to be key structural similarities between these inhibitors that could be incorporated into SWH to target BACE in cells. Additionally, their small molecule nature represents a significant difference from the peptidic construct of SWH.

Peptide inhibitors are much more structurally related to SWH and constitute a more relevant class of compounds from which to model a cell-active prochelator. The first reported peptide BACE inhibitor was based off the APP substrate sequence and contained a statine moiety at the hydrolysis site. Later generation peptide inhibitors utilized hydroxyethylene transition state isosteres and even macrocycles to moderately improve the potency in vitro. Many of these peptide inhibitors, however, displayed little to no efficacy in cells, likely because of the cellular localization of active BACE enzyme. Even though BACE is expressed on the extracellular membrane of cells, it is inactive at pH 7.4 and is unlikely to cleave APP when exposed to the extracellular environment. To gain activity, BACE must be endocytosed to an endosome where ATPase pumps protons into the endosomal lumen and lowers the pH to between 4-5. Early peptide inhibitors could not access these intracellular compartments and thus were ineffective in cell systems. It is important to mention that peptide BACE inhibitors have now been developed that are effective in cells. However, these peptides contain almost entirely lipophilic residues in order to be cell permeable. This strategy is not easily amenable to SWH because the sequence has already been optimized not only to act as a BACE substrate, but also for the resultant chelator to have appropriate copper binding characteristics.

In 2008, Lawrence Rajendran and coworkers at the Max Planck Institute in Germany utilized a cell impermeable peptide inhibitor modified with a sterol anchor and polyethylene glycol (PEG) linker to yield a cell-active BACE inhibitor. The sterol effectively anchors the peptide to lipid raft domains in the cell membrane and renders
the peptide capable of endocytosis to interact with active BACE enzyme. The PEG linker provides the correct spacing and orientation for the inhibitor to access the active site of the enzyme in a similar fashion as the native substrate APP. The construct used for this inhibitor is directly amenable to SWH as it does not require alteration of the peptide sequence.

Just as important as the substrate being hydrolyzed by BACE is the assay used to observe this conversion. A popular BACE activity assay used in vitro utilizes a FRET substrate that increases its fluorescence emission when hydrolyzed by BACE. Effective BACE inhibitors therefore suppress the fluorescence turn-on effect. This approach remains the standard in assaying in vitro BACE activity and because this technique is amenable to high-throughput screening, it has been used extensively to identify BACE inhibitors. However, this assay cannot be used to study BACE activity in live cells because, similar to many of the peptide inhibitors, the commercially available FRET probes do not specifically access the endosomal compartments where BACE is active, and thus are ineffective. Chemical FRET probes capable of measuring enzymatic activity in live cells have been previously developed, but not for BACE. The only FRET probe developed for BACE in living cells involved genetically directing two green fluorescent protein variants separated by a BACE substrate sequence to the cell membrane. This probe does not allow for real-time observation of BACE activity and involves genetic incorporation of large bulky proteins that may impede BACE hydrolysis. Furthermore, this report concludes that BACE is active on cell membranes because that is where fluorescence is observed. However, the authors do not mention that in the acidic pH of an endosome, GFP fluorescence is significantly quenched and the design of the probe prevents observation of hydrolysis in these environments.
The typical method for evaluating BACE activity, and the potency of inhibitors, in cells is an enzyme-linked immunosorbent assay (ELISA). This assay often requires genetic manipulations of the cell lines, several costly antibodies, and takes multiple days, making it slow, expensive, and laborious. Additionally, because ELISA uses antibodies to isolate and quantify the large protein fragments resulting from BACE cleavage, it is not a viable option for detection of CP. CP is too small a fragment to be reliably and selectively targeted by an antibody. As an alternative to using antibodies, alkaline phosphatase (AP) can be genetically encoded to a β-sequence so BACE hydrolysis releases AP, that can easily be monitored through para-nitrophenylphosphate decay. This method also involves genetic manipulation and appendage of large proteins onto a small substrate sequence. As with genetic FRET methods, while this may be amenable to our prochelator strategy, it prohibits any temporal or spatial assessment of activation.

In summary, there are several examples of how to target peptide substrates/inhibitors to BACE in living cells including sterol anchoring and using hydrophobic sequences. These approaches could be employed in our prochelator strategy if the native SWH sequence is not hydrolyzed by BACE in cells. Unfortunately, none of the currently available assays for monitoring BACE activity have the desired properties and are easily applicable to our system. The lack of a suitable detection technique will require the development of new methodology to study the conversion of SWH to CP by BACE. Among the most promising assays for measuring enzyme activity in living cells is a chemical FRET-based approach as it has been successfully employed to study other proteases and the properties of a successful chemical probe have been outlined. Furthermore, the methodology to study conversion of SWH to CP could also be used to evaluate BACE inhibitors through the lack of CP production. If the assay
could yield spatial and temporal assessment of BACE activity without genetic manipulation of the cell line, this would represent a significant advancement not only towards a cell active prochelator, but also for investigations into BACE activity under various conditions and treatments.

3.2 Results and Discussion

3.2.1 SWH and CP in Cells

HeLa cells were used to evaluate the conversion of SWH to CP in cells. The HeLa cell line is an immortal line derived in 1951 from a cervical cancer patient named Henrietta Lacks. Though a cervical cancer cell line is not overly applicable to Alzheimer’s Disease, HeLa cells are remarkably robust, easy to culture, and most importantly, express appreciable levels of BACE. To optimize the methodology associated with converting SWH to CP by endogenous BACE, these cells were chosen over the more AD-like SH-SY5Y cells. As is discussed in the next chapter while SH-SY5Y cells are more neuronal in nature, they are more difficult to culture and express relatively little BACE enzyme.\textsuperscript{137} If activation to the chelator can be shown in HeLa cells, then an analogous treatment of SH-SY5Y cells under Alzheimer’s like conditions could be used to demonstrate the efficacy of SWH and CP in cell culture.

3.2.1.1 Toxicity and Stability of SWH and CP

In order to rescue cells from AD conditions, neither SWH nor CP should have appreciable toxicity. To assess the toxicity of both peptides, HeLa cells were plated in 96-well plates and treated with various concentrations of each peptide from 0-100 µM for 24 hours and assayed via the LDH cytotoxicity assay. As demonstrated by the Davidson and NC State red bars in Figure 23 and Figure 24, both peptides are non-toxic to HeLa cells in the range of concentrations tested. This result is encouraging, albeit
somewhat expected, as both compounds are short peptides composed of naturally occurring amino acids.

![Figure 23: SWH Toxicity in HeLa Cells](image)

![Figure 24: CP Toxicity in HeLa Cells](image)

HeLa cells plated at 10,000 cells/well and incubated with indicated amounts of CP and SWH for 24 hours in MEM. 1% Triton X-100 was used as negative control.  n = 3 for each condition. Toxicity assayed with LDH cytotoxicity assay where a higher Abs @ 490 is indicative of more cell death.

The peptides were also analyzed via LC-MS after 24-hour incubations in minimal essential media (MEM) and MEM with HeLa cells to assess their solution
stability. This experiment marked a first attempt at visualizing conversion of SWH to CP in the presence of cells. As shown in Table 5, complete recovery of both peptides was achieved when incubated in MEM, but in the presence of HeLa cells, only about 90% of each peptide was recovered. The results suggest that cells are responsible for internalizing or degrading the peptides in some fashion. SWH degradation in the presence of cells is encouraging and suggests possible conversion to the chelator. This hypothesis was not supported by the LC-MS chromatogram, however, which did not contain any peaks corresponding to CP. Additionally, the similar rate of CP degradation is unwanted and implies a certain amount of non-specific proteolysis. It is possible that the cells are internalizing and digesting the peptides for their amino acids, as MEM is a poor culture media and starves cells of many essential nutrients. Alternatively, extracellular matrix proteins released from the cells may also be nonspecifically degrading the compounds. An important factor to consider in this experiment is the limit of detection of LC-MS. If the chelator is formed in small amounts (< 10 μM), it would be very difficult to detect over baseline noise in both the UV and mass spectrum detectors. The need for more sensitivity necessitated the development of an alternative assay for detecting CP in cell culture media.

Table 5: SWH and CP stability

<table>
<thead>
<tr>
<th>24 hour incubation</th>
<th>SWH Recovered</th>
<th>CP Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>100%</td>
<td>97%</td>
</tr>
<tr>
<td>MEM with HeLa cells</td>
<td>92%</td>
<td>89%</td>
</tr>
</tbody>
</table>

3.2.1.2 Cell Supernatant Calcein Assay

A more sensitive assay than LC-MS for detecting CP in cell culture media was developed using principals similar to the calcein assay described in Chapter 2. We have already demonstrated that, once formed, CP is able to strip copper from calcein and
restore fluorescence. This provides a very sensitive readout and the capability to detect CP in solution even at concentrations \( \leq 1 \text{ \textmu M} \). To provide maximal sensitivity while avoiding inner filter effects, the linear range of calcein fluorescence was determined in MEM. As can be seen in Figure 25, calcein fluorescence increases linearly until the concentration reaches 5 – 10 \( \text{\textmu M} \) after which, it begins to plateau due to self-quenching. From Figure 25 it was concluded that a calcein concentration of 5 \( \text{\textmu M} \) would be sufficient as a fluorescence indicator.

![Figure 25: Calcein Inner Filter Effects](image)

Calcein dissolved in 300 \( \mu L \) MEM in a 96-well plate at indicated concentrations. Fluorescence recorded on plate reader \( \lambda_{ex/em} = 490/535 \text{ nm} \)

In contrast to previous experiments where calcein fluorescence is rapidly and efficiently quenched by copper in HEPES buffer (Figure 19), the same copper-dependent quenching was not observed in MEM. It was concluded that because calcein is not a copper specific molecule, the high concentration of salts in MEM, in excess of 100 mM for NaCl, were interfering with copper binding. Preincubation of copper with calcein in water and subsequent dilution into MEM also resulted in immediate displacement of the...
metal cation and a similarly unquenched fluorescence signal. To overcome this problem, more copper-specific fluorescent molecules, phen-green and FloS, were employed (Figure 26). Unfortunately, while both of these indicators displayed efficient copper-induced fluorescent quenching in MEM, CP was unable to restore the fluorescence signal when added to the quenched solution, rendering these molecules ineffective.

![Phen-Green](image1.png) ![FloS](image2.png)

**Figure 26: Fluorescent copper chelators**

We hypothesized that the salts in MEM were responsible for preventing copper from quenching the fluorescence of calcein. Therefore, in order to use calcein as an indicator, the salts must be removed from the cell culture media. This must be done after incubation with HeLa cells because cells require high salt levels to retain osmotic equilibrium. To remove the salts while retaining the peptides, 200 µL pipette tips fitted with a small plug of C18 packing material called Zip-Tips were employed. By drawing the MEM, along with any peptides in the media onto the Zip-Tip, and aspirating with water, we were able to wash away salts while the biological material was retained on the packing. Subsequent aspiration with 80% acetonitrile eluted the peptides from the C18 packing in the absence of salts. This approach has the added benefit of concentrating the peptides contained in the cell media up to 2-times due to the low volume of acetonitrile required to elute the peptides from the tip. It should be mentioned that 100% recovery
of the material is not possible, and in fact, recovery studies with CP demonstrated only about 70-80% of the chelator could be eluted from an average Zip-Tip. This percentage is still high enough given the sensitivity of calcein fluorescence, but necessitates the use of a calibration curve for accurate quantitation of CP. Finally, addition of a quenched calcein-copper stock solution in HEPES buffer to the eluent resulted in an increasing fluorescence response indicating the amount of CP in the media. The results shown in Figure 27 demonstrate the effectiveness of the described calcein assay, as well as the effect of incubating SWH with HeLa cells.

![Figure 27: SWH with Cells analyzed via Calcein Assay](image)

CP controls (0, 2, 4, 6, 8, 10 µM) and SWH (100 µM) added to HeLa cells and incubated for 24 hours. CP (5 µM), SWH (100 µM), and MEM without incubation with cells constituted positive control, negative control, and blank respectively. All solutions filtered through Zip-Tip and analyzed via calcein assay on plate reader $\lambda_{ex/em} = 490/535$ nm. SWH incubations did not induce fluorescence response indicating a lack of chelator product in the extracellular media.
Various concentrations of CP (0 – 10 µM) were spiked directly into the cell media and analyzed after a 24-hour incubation in the presence of HeLa Cells. As expected, the fluorescence response increases linearly with the concentration of CP in the media. Positive and negative controls in which 5 µM CP or 100 µM SWH was added to the media after it was removed from cells were also analyzed (Duke-blue bars). Disappointingly, cells incubated with 100 µM SWH did not induce a fluorescence increase in the calcein assay indicating it was not converted to the chelator product after 24 hours (Carolina-blue bar).

Longer incubation times of 2 - 4 days were evaluated to try and increase the amount of CP produced, however Figure 28 demonstrates that after 2 days in MEM, the cells have completely digested the CP controls with only a small amount of the 10 µM condition remaining. CP loss most likely indicates the cells are deprived of nutrients and require the amino acids constituting CP. Not surprisingly, there was no additional fluorescence response in the SWH-treated wells over time as the prochelator was probably non-specifically degraded as well, and not converted to the chelator CP.
Figure 28: CP and SWH in MEM with HeLa cells

CP controls (0, 2, 4, 6, 8, 10 µM) and SWH (100 µM) added to HeLa cells and incubated for 1-4 days. CP (5 µM), SWH (100 µM), and MEM without incubation with cells constituted positive control, negative control, and blank respectively. All solutions filtered through Zip-Tip and analyzed via calcein assay on plate reader $\lambda_{ex/em} = 490/535$ nm. Lack of fluorescence response in days 2-4 most likely indicates non-specific digestion of peptides due to depleted nutrients in the culture media.

OptiMEM was used as an alternative to MEM because OptiMEM contains more than twice the amount of nutrients in MEM, and can increase the longevity of treated cells. Figure 29 suggests that cells cultured in OptiMEM do not degrade CP as rapidly as those cultured in MEM. However, after 3 days, even cells cultured in OptiMEM fail to give a strong fluorescence response for CP controls in the calcin assay. Similar to the MEM trials, experiments performed with SWH in OptiMEM also showed no fluorescence response when incubated with cells indicating a lack of chelator formation. These data are in agreement with the LC-MS data obtained during the stability study and indicate that SWH, in its current form, cannot be converted to CP by endogenous cellular BACE.
CP (0, 2, 4, 6, 8, 10 µM) added to HeLa cells in OptiMEM and incubated for 1-3 days. OptiMEM without incubation with cells constituted blank. All solutions filtered through Zip-Tip and analyzed via calcein assay on plate reader λ<sub>ex/em</sub> = 490/535 nm.

### 3.2.2 Lipid Raft Targeting

In its original form SWH is not a substrate for BACE in cells. As mentioned in the background section, this is largely due to the cellular localization of active BACE enzyme. While BACE is expressed on the cell membrane, the enzyme is only active in acidic intracellular vesicles. Without targeting SWH to these vesicles, BACE hydrolysis cannot occur.

It has been reported that BACE expressed at the cell membrane is trafficked to microdomains known as lipid rafts. These domains contain a high concentration of cholesterol and sphingolipids in comparison to the rest of the membrane. By attaching a sterol to the compound of interest, lipid rafts can be effectively targeted in cell systems. This strategy has been used previously to localize antioxidants and even a peptide BACE inhibitor to lipid raft domains. We sought to employ the same cholesterol targeting strategy to make SWH a suitable substrate for BACE in cells. To do this, we
first appended a dihydrocholesterol group to the side chain of aspartic acid through an ester linkage, and incorporated this modified amino acid onto the C-terminus of SWH. Then, the N-terminus was fluorescently tagged with fluorescein (termed F-SWH-C), incubated with HeLa cells, and imaged via fluorescence microscopy in order to determine if the cholesterol effectively anchors the prochelator to cell membranes. The results in Figure 30 demonstrate that fluorescently tagged SWH is effectively anchored to the cell membrane by the cholesterol moiety (bottom panel). Control experiments with fluorescently tagged SWH that does not contain the cholesterol anchor (F-SWH) show no response under the microscope (top panel).

![Chemical structures and images]

**Figure 30: Targeting SWH to cell membranes via dihydrocholesterol moiety**

Fluorescent substrates incubated with HeLa cells for 3 hours at 37°C. Treatments were removed and replaced with fresh OptiMEM. Cells immediately imaged on Axio Observer microscope using the GFP filter cube ($\lambda_{ex/em} = 470/525$ nm) at 60X magnification. Strong signal observed with cholesterol-containing substrate indicates effective cellular targeting.

Figure 30 also confirms the results from the calcein assay discussed earlier. Without purposefully targeting SWH to cells, the substrate does not interact with cell
membranes nor is it capable of being endocytosed. As endocytosis is a prerequisite for BACE hydrolysis, it is not surprising that the native SWH peptide is unable to be converted to CP in cells.

To further clarify the precise localization of F-SWH-C on the membrane, lipid raft domains were stained by tagging raft-specific protein GM1 with AlexaFluor 598, followed by imaging using dual fluorescence confocal microscopy. Figure 31 shows that when HeLa cells are co-stained for lipid rafts (A) and F-SWH-C (B), the points of greatest localization of F-SWH-C overlay with lipid raft marker GM1 (orange spots in C). It is important to notice the cholesterol also anchors SWH to non-raft domains, but is not as concentrated in these areas.

![Image](image.png)

**Figure 31: F-SWH-C and lipid rafts imaged via confocal microscopy**

Lipid rafts of HeLa cells stained with AlexaFluor 598 (A) followed by 3-hour incubation with F-SWH-C (B) and imaged via dual fluorescence confocal microscopy. Overlay (C) indicates F-SWH-C is concentrated in lipid raft domains.

Total internal reflection fluorescence (TIRF) microscopy was used to further confirm SWH localization to lipid rafts was occurring at the membrane and not intracellularly. TIRF microscopy uses an evanescent excitation wave that decays exponentially from the source to selectively illuminate only a thin section (~100 nm) of tissue directly in contact with the glass slide. This type of microscopy allows for
selective excitation of the basal cell membrane, as well as a small slice of the cytoplasm instead of the whole cell. Even though the images in Figure 32 are not as clear as the confocal images, an overlay between lipid rafts and F-SWH-C can be observed, indicating co-localization is likely occurring on the membrane.

![Figure 32: TIRF microscopy of F-SWH-C and lipid rafts](image)

Lipid rafts of HeLa cells stained with AlexaFluor 598 followed by 3-hour incubation with F-SWH-C and imaged via TIRF microscopy at 100X magnification. Overlay indicates F-SWH-C and lipid rafts are co-localizing on the cell membrane.

The results from the lipid raft studies have demonstrated important structural requirements for designing a viable BACE-activated prochelator in cells. Unmodified peptide substrates do not interact with endogenous BACE at the cell membrane and require an incorporated targeting vector. Additionally, dihydrocholesterol is an
effective component for directing SWH, not only to the cell membrane, but more specifically, to lipid raft domains where BACE is thought to reside. Finally, even though this localization appears to be membrane specific, whether or not the cholesterol renders SWH capable of endocytosis and hydrolysis by BACE has yet to be demonstrated.

3.2.3 Cholesterol Anchored Prochelators in the Calcein Assay

Cholesterol anchoring provides a suitable targeting vector to bring SWH to lipid raft domains for BACE hydrolysis. With this information, we synthesized two modified SWH substrates to evaluate BACE-mediated CP formation via the calcein assay. One substrate, called C-term, contains the cholesterol anchor appended to the C-terminus of the SWH substrate. Similarly, N-term contains the cholesterol on the N-terminus of the substrate. The two modified BACE substrates are shown in Figure 33.

Figure 33: Cholesterol-modified BACE substrates
Evaluation of C-term via the calcein assay proved inconclusive. Anchoring the substrate to the cell membrane via the C-terminus results in CP, if formed, also anchored to the cell membrane. As the calcein assay is predicated on CP being released into the extracellular media, this assay would not be able detect CP still attached to the cell. Additionally, control experiments indicate that cholesterol anchored peptides are very difficult to elute from the C18 packing material used in the Zip-Tip desalting step. Therefore, even if the cholesterol-containing product were released from the cell, most of the chelator would be retained on the Zip-Tip. In fact, C8 columns had to be used for analytical and semi-prep HPLC experiments with the cholesterol substrates because the analytes would not elute from a C18 column. Rather than developing additional analytical methodology to assess C-term hydrolysis, this peptide was abandoned in light of a recent publication that suggested the anchor must be separated from the substrate by a suitable length linker in order to be properly oriented for BACE hydrolysis.\textsuperscript{129} Linker involvement seems intuitive as the natural BACE substrate, APP, is not cleaved directly on the cell membrane but rather contains several amino acid residues between the membrane domain and the BACE hydrolysis site.

Calcein experiments evaluating the N-term substrate also yielded negative results. It is expected that upon BACE hydrolysis, CP would be released from the cell membrane, however no CP was detected in the extracellular media as shown by Figure 34. Again, this is not surprising given N-term does not contain a linker between the cholesterol and the substrate. Additionally, anchoring the substrate to the membrane by the N-terminus orients the C-terminus towards the extracellular environment. This effectively reverses the peptide chain direction as compared to the native substrate APP, in which the N-terminus extends away from the membrane. Experiments using a
cholesterol-anchored BACE inhibitor demonstrate that anchoring via the N-terminus does not yield a competent inhibitor even with a linker present.\textsuperscript{130}

![Figure 34: N-term calcein assay results](image)

CP controls (0, 2, 4, 6, 8, 10 µM) and N-term (100 µM) added to HeLa cells and incubated for 24 hours. CP (5 µM), N-term (100 µM), and OptiMEM without incubation with cells constituted positive control, negative control, and blank respectively. All solutions filtered through Zip-Tip and analyzed via calcein assay on plate reader $\lambda_{ex/em} = 490/535$ nm.

These results demonstrate the need for additional functionality to be built into the SWH substrate. A suitable linker must be incorporated between the substrate and the cholesterol anchor. Additionally, the cholesterol anchor must be attached on the C-terminus to maintain proper substrate orientation. This last point has several important consequences moving forward. Using a C-terminal anchor construction, the resultant chelator will be permanently attached the cell membrane. A membrane associated chelator may be advantageous for an Alzheimer’s Disease drug as lipid membranes are one of the primary targets of Aβ related toxicity.\textsuperscript{141} Also, lipid rafts have been
demonstrated to facilitate Aβ oligomerization. Therefore, specific targeting to these areas may enhance the efficacy of the chelator as opposed to a free peptide. However, a C-terminally anchored peptide requires alternative methodology to detect BACE hydrolysis, as the calcein assay will not work for the reasons discussed above.

### 3.2.4 SWH-FRET Constructs

Traditional ELISA methods for detecting BACE hydrolysis in cells are not applicable for detecting conversion of SWH to CP. Similarly, the previously described calcein assay is not appropriate if the resultant chelator is attached to the cell membrane. Therefore, an approach based on live-cell microscopy and FRET was initiated. This strategy uses a fluorescent chromophore attached to one end of the SWH peptide and a quenching group attached to the other end. In the intact state, the fluorescence of the chromophore is highly diminished because of intermolecular FRET. Upon hydrolysis, the chromophore is no longer covalently bound to the quenching group, resulting in a turn-on effect of the chromophore. Fluorescence turn-on can be monitored in vitro by a plate reader, or, as we intend, in live cells via microscopy. FRET probes have been used extensively in the literature to evaluate the in vitro activity of a variety of enzymes, including several BACE-specific examples. FRET probes have also been used in living cells with the turn-on effect observable by fluorescence microscopy.

The first generation SWH-FRET probe utilized in our lab called MCA-SWH-DNP (Figure 35), contained a 7-methoxycoumarin fluorophore attached to the N-terminus, which was quenched by a dinitrophenyl group bound to the ε-amine side chain of lysine on the C-terminus.
This probe was effectively cleaved by BACE and produced a fluorescence turn-on effect in vitro, but would not make a suitable probe for fluorescence microscopy because the excitation/emission wavelengths of methoxycoumarin (323/395 nm) are too short for live cell microscopy. Traditional microscopes are fitted with a DAPI filter with excitation/emission = 390/435 nm. This filter would not effectively excite the methoxycoumarin, nor would the emission be detectable above baseline noise. For microscopy purposes, a suitable donor/acceptor pair ideally has longer excitation and emission wavelengths. Visible excitation wavelengths also avoid toxicity issues arising from UV irradiation, while visible emission decreases background noise from cellular autofluorescence.

To satisfy these requirements a second-generation FRET probe called Rho-SWH-Fluor was synthesized using fluorescein and rhodamine molecules for the donor and acceptor respectively (Figure 36). The fluorescein is bound to the side chain of lysine on the C-terminus through an amide linkage while the rhodamine is attached to the N-terminus through a thiourea.
This probe offered excellent excitation/emission properties (485 / 520 nm) and had the capability of being a ratiometric sensor as both chromophores are fluorescent. However, it had several crippling limitations. While FRET does occur between this pair, rhodamine does not quench the fluorescence of fluorescein to a great degree (3-fold). Hence the turn-on effect induced by hydrolysis is not very dramatic. Furthermore, both of these fluorophores are pH sensitive. Acidic pH, such as that present in an endosome, induces the free carboxylic acid in each chromophore to cyclize to a lactone ring that severely decreases fluorescence. Most importantly, however, Rho-SWH-Fluor is not efficiently hydrolyzed by BACE. When this substrate is incubated with BACE for 24 hours and analyzed via LC-MS, virtually no product is observed. This is confirmed by in vitro fluorescence data, which shows virtually no change after 24-hour incubation with BACE (not shown).

We have already demonstrated through studies with D-amino acids and alternative substrates, that BACE is moderately specific in its amino acid preference. Additionally, the fact that the first generation methoxy-coumarin FRET substrate was hydrolyzed by BACE, while the second generation rhodamine version was not, lends
support to the hypothesis that stericus are playing an important role in enzyme recognition. As mentioned in Chapter 2, BACE recognition is more heavily dependent on the N-terminal residues than the C-terminal ones. The C-terminus already contains a mass tag that is tolerated by BACE and the chromophore is attached even further outside the active site. Therefore, we believe the size of the chromophore on the N-terminus determines whether or not the substrate can be hydrolyzed by BACE.

With these considerations in mind, we synthesized a FRET substrate that contained a dimethylaminocoumarin (DMACA) fluorophore on the N-terminus and a 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL) group on the C-terminus called DMACA-SWH-DABCYL (Figure 37).

This pair was found to be an appropriate donor/acceptor pair for multiple reasons. The DMACA fluorophore has excitation/emission properties (370/480 nm) that are compatible with live cell fluorescence microscopy. Though ideal, longer excitation wavelengths come at the price of steric bulk, which needs to be avoided in this case. A large Stokes shift yields an emission wavelength sufficiently long enough to use a cyan fluorescent protein (CFP) filter in the microscope. This eliminates a majority of the
background signal from cell autofluorescence, which would otherwise be observed with a DAPI emission filter. The DABCYL group on the C-terminus has a large absorbance band between 420 - 520 nm and is a “dark quencher,” meaning that it does not have an inherent fluorescence signal of its own. This eliminates the possibility of the probe being ratiometric, but for live cell microscopy, this may be advantageous in avoiding signal overlap between the two fluorophores. Furthermore, as can be seen from Figure 38, the DMACA group is not sensitive to the acidic pH of an endosome as it fluoresces similarly at both pH 7.4 and 4.5 (dashed lines). This fluorescence is greatly diminished when covalently bonded to the DABCYL group via the SWH substrate in DMACA-SWH-DABCYL (solid lines).

Figure 38: DMACA/DABCYL fluorescence properties

DMACA (5 µM) and DABCYL (5 µM) dissolved in 0.1M sodium acetate buffer pH 4.5 or 20 mM HEPES buffer pH 7.4 have similar fluorescence properties (dashed lines) indicating DMACA is equally sensitive at either pH. The fluorescence of DMACA-SWH-DABCYL (5 µM) is significantly quenched in both buffers due to intermolecular FRET (solid lines). $\lambda_{ex} = 370$ nm.
Importantly, the DMACA-SWH-DABCYL sequence was found to be a suitable substrate for BACE. Incubation of the FRET probe with BACE for 24 hours resulted in a 40% production of the correct product as monitored by LC-MS. This is only slightly less than the native SWH peptide without the chromophores (50%). Hydrolysis also induces a significant fluorescence turn-on from the DMACA as monitored on the fluorometer and plate reader. It should be noted that the placement of the DMACA group on the N-terminus is not only for synthetic ease, but also to ensure it is not quenched by liberation of the chelator and subsequent copper binding. We have already demonstrated that copper binding to CP quenches the fluorescence of tryptophan, and if DMACA were conjugated to the C-terminus, it would be covalently bound to the resultant copper chelator and susceptible to quenching in cells.

3.2.5 PEG Linkers

Literature reports suggest that in order to effectively situate a substrate for BACE hydrolysis in cells, a linker is necessary to separate the membrane anchor domain (cholesterol) from the substrate sequence. Attachment of the linker between these groups forces the substrate peptide to be oriented from the C-terminus to the N-terminus as it extends away from the cell membrane analogous to APP. Polyethylene glycol (PEG) is suitable for this purpose for multiple reasons. PEG is metabolically stable, increases aqueous solubility, and does not induce an immune response. Furthermore, the length can be easily altered to achieve an ideal spacer. The initial attempt at incorporating a spacer utilized a 24-mer PEG unit with a free carboxylic acid on one end and an FMOC-protected amine on the other. Synthetic efforts to couple the linker to the cholesterol-modified aspartic acid on the peptide synthesizer were unsuccessful. It is possible that a linker of this length is prone to folding and may hinder the carboxylic acid from being activated by HBTU, or prevent subsequent coupling to
the free amine on the solid support. We concluded that incorporating the entire linker in a single step was not the best approach because the long PEG linker is very expensive and the reaction cannot be monitored effectively. Instead, we built the linker stepwise using sequential coupling of 4- and 5-mer PEG units. This was far more cost effective and allowed for the reactions to be checked at each coupling until the entire spacer was completed.

The length of the PEG chain was carefully chosen. APP contains 28 residues between the BACE cleavage site to the cell membrane. The PEG linker provides for the equivalent of 29 residues between the membrane anchor and the hydrolysis site. Recent reports have suggested that the length of the linker is not strictly important, but that too short a linker could alter specificity or prevent hydrolysis.

With the cholesterol anchor coupled to an appropriate linker, the DMACA-SWH-DABCYL substrate was added to yield a first generation FRET-PEG-Anchor (FPC) compound for studying BACE hydrolysis.

3.2.6 FPC

The structure of the first generation FRET probe (termed FPC) for cellular BACE activity is presented in Figure 39. FPC contains all of the elements discussed in previous sections. The DMACA fluorophore is separated from its quenching DABCYL group by the SWH substrate. Sequential PEG linkers and finally the cholesterol membrane anchor follow this sequence to yield the full probe.
It has already been determined that the DMACA-SWH-DABCYL FRET construct is a suitable BACE substrate. Disappointingly however, attachment of the PEG and cholesterol groups rendered FPC unable to be cleaved by BACE as monitored by LC-MS. Lack of hydrolysis was confirmed by fluorescence data presented in Figure 40. When DMACA-SWH-DABCYL is incubated with BACE and the fluorescence is monitored over several hours, an increasing fluorescence is observed due to liberation of the DMACA group upon BACE hydrolysis (blue diamonds). Control experiments using the same substrate in the absence of BACE does not yield a turn-on response (black squares). FPC in the presence of BACE closely follows the control experiment indicating hydrolysis is not occurring (green circles).
Figure 40: FPC with BACE in vitro

DMACA-SWH-DABCYL (4 μM) and FPC (4 μM) diluted in 200 μL 0.1 M sodium acetate buffer pH 4.5 (0.2-0.4% DMSO) at 37°C. 5 μL BACE added to initiate reaction and fluorescence read on plate reader every hour for 5 hours. $\lambda_{ex/em} = 490/535$ nm. FPC is not a BACE substrate as indicated by lack of fluorescence turn-on.

It was observed under the bright field microscope that aggregates were forming in solution upon diluting FPC dissolved in DMSO into pH 4.5 sodium acetate buffer. Further investigation into the aggregates using dynamic light scattering revealed that even though FPC is soluble in DMSO, when diluted into aqueous solution, the compound immediately precipitates and forms aggregates with a wide distribution of sizes between 100 and 1000 nm (Figure 41).
Figure 41: FPC aggregation observed by dynamic light scattering

FPC (4µM) diluted in 0.1M sodium acetate buffer pH 4.5 in a 1 cm cuvette. Aggregation monitored via dynamic light scattering. Results indicate a polydistribution of aggregates across a wide range of sizes.

We concluded that because FPC was immediately precipitating from solution, it was unable to be hydrolyzed by BACE. Additionally, we hypothesized that the hydrophobic cholesterol group may be folding into a hydrophobic region of the SWH peptide, whereby even if a fraction of FPC remained in solution, it may too hindered to undergo hydrolysis. Therefore, in order to increase the solubility of the probe and prevent cholesterol from interfering in hydrolysis, an additional arginine was introduced into the sequence.
3.2.7 β-MAP

An additional arginine residue was introduced adjacent to the cholesterol anchor and before the PEG linker to yield the second-generation β-Secretase membrane anchored probe called β-MAP. The positively charged guanidinium side chain of arginine not only introduces additional polarity to aid solubility, but at pH 4.5, it prevents the cholesterol from folding into the substrate sequence through electrostatic repulsion with the positively charged histidine and arginine residues of SWH. The structure of β-MAP is shown in Figure 42.

![Structure of β-MAP](image)

**Figure 42: Structure of β-MAP**

Dynamic light scattering was again utilized to determine if β-MAP is soluble in aqueous solution. As can be seen in Figure 43, β-MAP diluted into pH 4.5 sodium acetate buffer has a very narrow distribution of particles around 10 nm. This data is consistent with free peptides in solution and in stark contrast to FPC, which formed a polydistribution of large aggregates.
β-MAP (4µM) diluted in 0.1M sodium acetate buffer pH 4.5 in a 1 cm cuvette. Aggregation monitored via dynamic light scattering. Results suggest a nearly homogeneous distribution of small particles indicative of free species in solution.

With this final design modification, we have created a FRET probe that is specifically engineered to target BACE in living cells. Each component outlined above was carefully considered and implemented in response to directly observable experimental data and literature precedence. The following sections detail experiments to evaluate the performance of β-MAP in living cells.

3.2.8 β-MAP in vitro

β-MAP can be used to monitor in vitro BACE hydrolysis analogous to many other FRET probes reported in the literature. As shown in Figure 44, an in vitro evaluation of two commercially available BACE inhibitors (one small molecule, and one peptide, (Figure 56) yielded expected results. In the absence of inhibitor, a strong
fluorescence turn-on was observed when β-MAP was incubated with BACE corresponding to liberation of the chromophore from the quenching DABCYL group. The cleavage site was confirmed through identification of the products via LC-MS. When an inhibitor was added to the reaction mixture, the turn-on signal was significantly attenuated in both cases.

![Graph](image-url)

**Figure 44: β-MAP in vitro with BACE inhibitors**

β-MAP (10 µM) dissolved in 0.1 M sodium acetate buffer pH 4.5 in the absence or presence of BACE inhibitors Axon 1125 (10 µM) or β-secretase inhibitor III (10 µM). In the absence of inhibitors, addition of BACE (5 µL) induces a strong fluorescence turn-on over time as monitored by the plate reader ($\lambda_{ex/em} = 355/460$ nm) whereas inhibitors suppress this effect.

### 3.2.9 β-MAP in HeLa Cells

The novelty of β-MAP is in its applicability to live cell models. When β-MAP is incubated with live HeLa cells and observed via time-lapse fluorescence microscopy, a strong signal increase is observed in distinct parts of the cell. As mentioned previously,
the DMACA fluorescence signal is easily distinguishable from background noise and fluoresces strongly in acidic intracellular vesicles as well as the more neutral cytoplasm and extracellular space. The signal does not permeate the nucleus of the cell nor does it occur homogeneously across the cell membrane or in the cytoplasm. These observations indicate that hydrolysis is occurring in distinct compartments, which is consistent with the previously reported localization and mechanism of BACE. Figure 45 shows a sample time-lapse sequence demonstrating fluorescence the turn-on effect.
β-MAP (200 nM) incubated with HeLa cells in pH 4.5 OptiMEM for 10 min followed by replacement of the media with fresh OptiMEM. Images captured every 10 min on a fluorescence microscope using DAPI excitation (390 nm) and CFP emission (470 nm) at 60X magnification.

The toxicity of β-MAP to HeLa cells was also evaluated. As can be seen from Figure 46, cells treated with β-MAP have high viability (blue bars) and low toxicity.
(magenta bars) across the range of concentrations tested. TX-100 wells represent the negative control in which all cells are dead.

Figure 46: β-MAP toxicity study

HeLa cells plated at 10,000 cells/well and incubated with indicated amounts of β-MAP for 10 min in pH 4.5 OptiMEM. After incubation, media was replaced with fresh OptiMEM for 24 hours. 1% Triton X-100 was used as negative control. n = 3 for each condition. Toxicity assayed with LDH cytotoxicity and viability assayed with Cell Titer Blue reagent. β-MAP is nontoxic at the concentrations tested.

It should be noted that in addition to microscopy, we also attempted to observe the fluorescence turn-on by a plate reader assay. Unfortunately, we did not observe any difference between treated and untreated cells. The primary reason being the detector on the plate reader is not sensitive enough to detect concentrations in the low nanomolar range. Furthermore, the fluorescence turn-on is only occurring in distinct cellular compartments in a very narrow plane of focus. The plate reader is designed to measure through an entire sample and could not be properly focused to capture the fluorescence turn-on.
3.2.10 Quantitation of β-MAP Images

Quantitative analysis can be applied to the microscopy images by integrating the fluorescence density corresponding to β-MAP and plotting versus time. Raw images are pixilated using an appropriate minimum gray value threshold so as not to include cell auto-fluorescence or background noise. The threshold is kept constant for all images within each stack (set of images at one location over different time points), but vary slightly between stacks because of different focusing parameters. The threshold for each stack is determined at later time points (> 4 h) where the signal is brightest to ensure background fluorescence is not included in the integration. Analyzing the pixilated images using Fiji image software yields the area of all pixels above the preset threshold and their average gray value. Multiplying the total area by the average gray value gives the total integrated density of the fluorescence image. The number of cells in each image is important, as quantification of the overall fluorescence signal is dependent on the number of cells in view. Care was taken to ensure that each stack selected for processing contained about the same number of cells. Additionally, 20X magnification was used to encompass more cells per image and obtain more statistically relevant results. Finally, by plotting the total integrated density of each image versus its corresponding time point, a graphical representation of the fluorescence turn-on is attained. The quantification process is illustrated in Figure 47.
Raw fluorescence images are smoothed and pixelated using an appropriate minimum threshold to eliminate background noise. Pixelated images are then integrated to yield a total integrated fluorescence for each image. Plotting the integrated fluorescence versus the time at which the original image was taken yields a graphical representation of β-MAP fluorescence turn-on in cells.

**3.2.11 β-MAP Specificity for BACE**

It has been previously demonstrated that SWH is non-specifically degraded in blood serum. While the proteases responsible for this degradation were not identified, non-specific hydrolysis of β-MAP is a major concern in cells. To assess the degree to which non-specific proteolysis is responsible for the fluorescence turn-on in cells, several experiments were performed.

**3.2.11.1 siRNA**

Small interfering RNA (siRNA) was used to knockdown BACE expression in HeLa cells. As seen in Figure 48, efficient knockdown of BACE expression (siBACE) yielded a diminished fluorescence turn-on (black dashed line) when compared to untreated (mock) and scrambled (siMED) siRNA controls (solid blue lines). The inset shows western blot analysis of BACE expression with β-actin used as a loading control. The lighter BACE band for siBACE indicates partial knockdown of enzyme expression.

---

1 Sunhee Hwang performed the Western Blot analysis for this experiment
While this experiment demonstrates that some of the fluorescence turn-on effect is, indeed, BACE-mediated, it does not rule out the possibility of non-specific hydrolysis accounting for at least a portion of the signal.

Figure 48: siRNA effectively diminishes BACE activity

BACE expression in HeLa cells was partially silenced by treatment with BACE-specific siRNA for 2 days as measured by Western blot analysis. Monitoring BACE activity in siRNA treated cells with β-MAP (200 nM) yielded a decreased fluorescence turn-on as compared to untreated (Mock) and scrambled (siMED) controls.

3.2.11.2 Broadband Protease Inhibitor Controls

In order to gain a better understanding of the role non-specific hydrolysis plays in the fluorescence turn-on of the FRET substrates, studies were performed both in vitro and in cells using the broadband protease inhibitors and small molecule BACE inhibitor (Axon 1125) listed in Table 6. All inhibitors, with the exception of Axon 1125, were combined in a protease inhibitor cocktail (PIC) and added simultaneously to reaction
wells, whereas Axon 1125 was added independently. The in vitro data presented in Figure 49 highlight several interesting characteristics of the inhibitors and their interaction with BACE and the digestive enzyme trypsin.

**Table 6: Protease inhibitors used in specificity studies**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Function</th>
<th>Treatment Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEBSF</td>
<td>Serine Protease Inhibitor</td>
<td></td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Trypsin Inhibitor</td>
<td></td>
</tr>
<tr>
<td>Bestatin</td>
<td>Aminopeptidase Inhibitor</td>
<td></td>
</tr>
<tr>
<td>E-64</td>
<td>Cysteine Peptidase Inhibitor</td>
<td></td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Cysteine, Serine, Threonine</td>
<td></td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>Aspartyl Protease Inhibitor</td>
<td></td>
</tr>
<tr>
<td>Axon 1125</td>
<td>BACE-specific Inhibitor</td>
<td>Axon 1125</td>
</tr>
</tbody>
</table>

(PIC)
Figure 49: In vitro enzyme and inhibitor effects on DMACA-SWH-DABCYL

DMACA-SWH-DABCYL (4 µM) dissolved in 0.1 M sodium acetate buffer pH 4.5 in the absence or presence of protease inhibitors Axon 1125 (10 µM) or Protease Inhibitor Cocktail (PIC). The substrate can be hydrolyzed by BACE and trypsin whereas concurrent treatment with the appropriate inhibitor prevents hydrolysis in both cases. Interestingly, PIC, which contains an aspartyl protease inhibitor does not inhibit BACE hydrolysis.

We used the DMACA-SWH-DABCYL FRET substrate in vitro because it is more rapidly hydrolyzed by BACE than the full β-MAP substrate. As can be seen from the blue lines, when the substrate is incubated with BACE (solid blue line), or BACE with the protease inhibitor cocktail (dotted blue line), the rate of hydrolysis is nearly identical. This is expected because even though the PIC contains a broad spectrum aspartyl protease inhibitor, Pepstatin A is known not to inhibit BACE. Conversely, treatment with the BACE inhibitor Axon 1125 completely blocks the hydrolysis of the substrate and prevents fluorescence turn-on (dashed blue line).
DMACA-SWH-DABCYL incubated with trypsin also reveals an interesting trend. The substrate and trypsin alone (solid red line), or with Axon 1125 (dashed red line), produce a turn-on effect. This indicates that the substrate can be hydrolyzed by trypsin. Even though trypsin is produced in the pancreas, and is an unlikely candidate for non-specific proteolysis in HeLa cells, it underscores the observation that the FRET substrate is not entirely BACE-specific. No conclusions can be drawn from the rate of hydrolysis being slightly slower than that of BACE because the concentration of active trypsin is unknown in this experiment. Finally, concurrent treatment with trypsin and PIC, which contains multiple trypsin (serine protease) inhibitors, abolishes enzymatic activity (dotted red line).

The inhibitor treatments were also evaluated in HeLa cells via microscopy using β-MAP as the BACE substrate. As can be seen from Figure 50, cells treated with β-MAP alone produce a strong fluorescence turn-on effect (black). When PIC is added to the wells, the fluorescence response is attenuated to a small degree, but much of the fluorescence response is retained (blue). Axon 1125, which has demonstrated cellular efficacy,\textsuperscript{105} abrogates the fluorescence response to a much larger degree (red) than the broadband inhibitors. Finally, the combination of both PIC and Axon 1125 silences the fluorescence signal nearly entirely (purple). This result affirms that while there does appear to be non-specific proteolysis occurring in cells, this only accounts for a small percentage of the observed fluorescence turn-on. A majority of the hydrolysis of β-MAP occurring in HeLa cells appears to be BACE-mediated.
HeLa cells treated with β-MAP (200 nM), Protease Inhibitor Cocktail (PIC 0.1%) and Axon 1125 (2.5 μM) with fluorescence monitored over time with fluorescence microscopy ($\lambda_{ex/em} = 390/470$ nm). Images processed for total integrated density as described above. Results indicate non-specific hydrolysis to β-MAP is responsible for a portion of the observed fluorescence signal, but a majority of the fluorescence turn-on is BACE mediated.

3.2.11.3 Scrambled β-MAP

A scrambled FRET probe was also synthesized to further evaluate the specificity of β-MAP for BACE. β-MAP contains the peptide sequence E-V-N-L-D-A-H-F-W-A-D-R that is hydrolyzed by BACE between the leucine (L) and aspartic acid (D). The scrambled version has the sequence F-V-H-D-N-A-L-E-W-A-D-R, which contains the same amount and type of amino acids, but the enzyme recognition fragment is in scrambled order. The tryptophan tag (WADR) was unaltered because it does not participate in enzyme recognition. The FRET version of this peptide (DMACA-FVHDNALEWADR-DABCYL) was first synthesized to confirm the sequence is not a
BACE substrate. Analysis of the scrambled FRET peptide by plate reader and LC-MS confirmed the sequence is not hydrolyzed by BACE (not shown). The scrambled β-MAP probe containing the PEG linker and cholesterol anchor was synthesized and compared to β-MAP in the cell microscopy method. A diminished fluorescence turn-on of the scrambled probe compared to β-MAP indicates that the scrambled β-MAP probe is not hydrolyzed as efficiently as β-MAP in cells (Figure 51). This data supports previous observations that there is a small degree of non-specific hydrolysis, but that β-MAP is largely hydrolyzed by BACE in cells. The structure and characterization of both “scrambled FRET” and “scrambled β-MAP” are provided in Appendix A.

Figure 51: Scrambled β-MAP in HeLa cells

HeLa cells treated with β-MAP or scrambled β-MAP (200 nM) with fluorescence monitored over time with fluorescence microscopy (λ<sub>exc/em</sub> = 390/470 nm). Images processed for total integrated density as described above. Results demonstrate the fluorescence turn-on of the scrambled sequence is not as pronounced as β-MAP indicating the scrambled sequence is not hydrolyzed as efficiently as β-MAP.
3.2.12 BACE Inhibitor Axon 1125

To further explore the utility of β-MAP and the associated microscopy method, HeLa cells were treated with varying concentrations of both the peptide BACE inhibitor and Axon 1125 small molecule inhibitor utilized in the in vitro BACE experiments (Figure 44). Though both inhibitors are effective in vitro, only Axon 1125 has cellular efficacy, as the peptide is unable to access the endosomal compartments where BACE is active. Qualitative analysis of the fluorescence images shows that when cells are treated with Axon 1125, a dose dependent decrease in the fluorescence signal is observed (Figure 52). Conversely, when cells are treated with the peptide BACE inhibitor, the fluorescence intensity is relatively constant across all doses tested, indicating it is not an effective inhibitor of BACE activity in cells (not shown).
Figure 52: HeLa cells treated with β-MAP and Axon 1125

Top Row: HeLa cells treated with β-MAP (200 nM) show a fluorescence turn-on over time under the fluorescence microscope at 20X magnification ($\lambda_{ex/em} = 390/470$ nm). Rows 2 and 3: Treatment with β-MAP in combination with BACE inhibitor Axon 1125 (5 nM, 2.5 µM) reduces fluorescence turn-on in a dose-dependent fashion.

By quantifying the fluorescence in each image as has been previously described, progress curves were created to graphically represent the dose dependent decrease in the fluorescence signal with increasing inhibitor concentration. Figure 53 shows the progress curves for selected concentrations of Axon 1125. By calculating the slope of each curve from 100 to 150 minutes, a rate of hydrolysis can be determined at each inhibitor concentration. As can be seen from Figure 54, the rate of fluorescence turn-on decreases with increasing inhibitor concentration.
Figure 53: Progress curves for HeLa cells treated with BACE inhibitor Axon 1125

HeLa cells treated with β-MAP (200 nm) and varying concentration of BACE inhibitor Axon 1125 (0-2.5 µM) and imaged via fluorescence microscopy every 10 min for 5 hours. ($\lambda_{ex/em} = 390/470$ nm). Progress curves represent the average fluorescence turn-on within each treatment condition. Error bars omitted for clarity. Axon 1125 produces a dose-dependent decrease in the fluorescence turn-on.
HeLa cells treated with β-MAP (200 nm) and varying concentration of BACE inhibitor Axon 1125 (0-500 nM) and imaged via fluorescence microscopy every 10 min for 5 hours. ($\lambda_{ex/em} = 390/470$ nm). Rate of Hydrolysis determined by linear fit of the progress curve (Figure 53) from 100-150 min. n=3 for all conditions except 5 nM (*) n=1. Axon 1125 produces a dose-dependent decrease in the fluorescence turn-on.

It is important to note that even at high inhibitor concentration (500 nM), there is still observable fluorescence turn-on. As has been already discussed, this is most likely due to non-specific cleavage of β-MAP. Non-specific cleavage is further exacerbated when a higher concentration of probe is used. At 5 µM β-MAP, the fluorescence response is not nearly as attenuated in the presence of inhibitors as when 200 nM probe is used (not shown). In fact the BACE inhibitor from which β-MAP was modeled also
showed non-selectivity above 200 nM. Despite minor selectivity issues, a clear trend is observed in the progress curves and rate of hydrolysis when increasing amounts of BACE inhibitor are added to the wells. More information about Axon 1125 as well as better selectivity by β-MAP would need to be obtained in order to ascertain reliable kinetic data (i.e. IC_{50}), however β-MAP can be used to make qualitative evaluations of inhibitor efficacy.

3.3 Conclusions

We have demonstrated that SWH cannot be converted to CP in living cells unless appropriately modified with a membrane anchor and PEG spacer. However, even with these improvements, conversion can be done only at a very low dosage (200 nM) as increasing the concentration leads to enhanced non-specific cleavage. Furthermore, even though cells are treated with 200 nM prochelator, it is likely that not all of the compound binds to the cell membrane, and that which does bind, is not all converted to chelator because of non-specific cleavage. Therefore the actual concentration of chelator formed on the cell membrane is likely to be in the low nanomolar range. This poses a great challenge to the original hypothesis of using the chelator to rescue cells from AD-like conditions. Unless a cell model that is extremely sensitive to Aβ(Cu) can be developed, this concentration of chelator is unlikely to improve the viability of cells under AD conditions.

However, labeling cells with β-MAP does represent a novel method for evaluating BACE activity in living cells. The design of the probe allows it to concentrate in lipid raft domains within the cell membrane and be cleaved by BACE in the same way as its natural substrate APP. Hydrolysis produces a fluorescence turn-on signal that can be easily monitored via fluorescence microscopy. This strategy permits identification of not only where BACE hydrolysis is occurring, but to what extent in real time. β-MAP
was used to verify Axon 1125 is a cellular BACE inhibitor, and because of its ease of use and applicability to unaltered cells, could be used to efficiently screen libraries of other potential BACE inhibitors.

While not part of the original hypothesis, the applications for a probe like β-MAP are very important. Currently, we are using β-MAP to evaluate how transient external stimuli may affect BACE activity without changing the enzyme’s expression levels. This has particular relevance at the synapse, which experiences acute neurotransmitter fluctuations and is also a purported site of accelerated Aβ accumulation and toxicity. This type of analysis would be very difficult using traditional ELISA methods. Multifunctional chemical tools such as β-MAP that can expedite research and deepen our understanding of the molecular players involved in AD play a crucial role in identifying new targets and developing therapeutics to combat this debilitating disease.

3.4 Experimental

3.4.1 Materials and Instrumentation

All chemicals and solvents were obtained from Sigma-Aldrich and used without further purification unless otherwise noted. All water was nanopure. Peptides were synthesized on a Protein Technologies PS3 automated peptide synthesizer and purified with a Waters 600 HPLC. Liquid chromatography-electrospray mass spectrometry (LC-MS) data were collected on an Agilent 1100 Series HPLC in line with a LC/MSD trap and a Daly conversion dynode detector. Zip-Tips were purchased from Millipore. In vitro BACE assays were conducted on a Perkin Elmer Victor 1420 plate reader. Fluorescence data were recorded on a FluoroLog-3 fluorimeter from HORIBA Jobin Yvon. Fluorescence microscopy was performed on a DeltaVision Elite deconvolution microscope with temperature, humidity, and CO₂ control. Lipid raft colocalization studies were performed on a Leica SP5 confocal microscope with a live cell chamber.
TIRF microscopy performed on a Leica AM TIRF MC microscope. All fluorescence images were processed using Fiji image software.

### 3.4.2 Preparation of Peptides

The precursor compound Fmoc-Asp(dihydrocholesterol)-OH was synthesized by esterification of the side chain of Fmoc-Asp-OtBu (Novabiochem) with 5α-Cholestan-3β-ol using MSNT / N-Methylimidazole in dichloromethane. Coupling was performed with dry solvents under argon. Deprotection with TFA yielded the Fmoc-Asp(Chol)-COOH building block. Fmoc-NH-(Peg)4-COOH (Novabiochem) was dissolved in DMF with sonication before use. Fmoc-Lys(DABCYL)-OH (AnaSpec) and DMACA (AnaSpec) were used without modification. For lipid raft studies, 5(6) carboxyfluorescein was coupled to the N-terminus of the peptide under standard conditions.

Peptides were synthesized on a 0.1 mmol scale on PAL-PEG-PS resin (Applied Biosystems). Standard Fmoc (9-fluorenylmethoxy-carbonyl)-protected natural and non-natural amino acids (Chem-Impex and Novabiochem) were coupled in 20 min cycles with HBTU (O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate) (Novabiochem) and N-methylmorpholine (NMM) (Acros) in N,N'-dimethylformamide (DMF) (Caledon). Fmoc protecting groups were removed by using 20% piperidine in DMF. Cleavage from the resin and removal of side chain protecting groups was accomplished by treating resin with a 10-mL mixture of 95% trifluoroacetic acid (TFA) and 2.5% triisopropylsilane (TIS) under nitrogen while shaking for 4 h. Peptide was precipitated from solution by evaporating off TFA with a nitrogen stream, followed by three washes with diethyl ether (Caledon). Purification was accomplished by semi-preparative reversed-phase HPLC on a Waters Spherisorb S10 C8 column with a linear 40 min gradient from 7 to 70% acetonitrile in water with 0.1% TFA. Purity was validated...
to be greater than 90% by analytical HPLC. Mass of each peptide was determined by ESI-MS. Peptides were stored as lyophilized powder at -20°C until use.

### 3.4.3 Cell Culture

Human cervical cancer cells, HeLa cells, were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate at 37 °C and 5% CO₂. To determine optimal plating density in a 96-well plate, the media was removed from the culture flask and cells were washed 1X with PBS. 1.5 mL trypsin was added and the flask was incubated for 5 min at 37 °C and 5% CO₂ to lift the cells. The lifted cells were removed and centrifuged at 800 rpm for 6 min at RT in 5 mL growth media. Media and trypsin were decanted and cells were resuspended in 2 mL fresh growth media. Cells were counted using a hemacytometer. Cells were plated in two 96-well plates at densities varying from 50 – 200,000 cells/well. Cells were allowed to adhere and incubated for 24 hours at 37 °C and 5% CO₂. After 24 hrs, Triton X-100 was added to a final concentration of 1% to one of the plates as a control. An LDH cytotoxicity assay was performed on both plates according to manufacturer’s instructions. This assay measures the amount of lactate dehydrogenase (LDH) released from dead cells via a colorometric reagent where a higher absorbance is indicative of more cell death. The results in Figure 55 show the total amount of possible cell death in the Triton X-100 plate (TX-100) and the amount of actual cell death in the untreated plate (Untreated) at each cell density. The inset shows a plot of the difference between the two conditions at each cell density (red line). The peak at 10,000 cells/well indicates optimal cell density. At concentrations lower than 10,000 cells/well there are not enough cells to generate a strong response in the LDH assay and at higher concentrations, the cells die from over crowding and nutrient depletion.
HeLa cells plated at varying densities via serial dilution in two 96-well plates. One plate was treated with 1% Triton X-100 to represent the total amount of LDH signal possible at a particular cell density. The other plate was treated with MEM as a positive control. The greatest difference in LDH activity between the two plates was seen at 10,000 cells/well which is indicative of the optimal cell density in a 96-well plate.

### 3.4.4 SWH and CP Toxicity

HeLa cells were plated in complete growth media in 96-well plates at 10,000 cells/well and allowed to adhere for 2 days. SWH and CP toxicity were evaluated by preparing 1 mL stock solutions of each peptide in MEM. Concentrations varied from 0 – 100 µM. Treatment conditions (200 µL) were added to HeLa cells in triplicate. 1% Triton X-100 in MEM was used as a positive control. Cells were incubated with the peptides for 24 hours after which, the media was removed and assayed for LDH activity according to manufacturer’s instructions. Toxicity data normalized to the TX-100 wells.
having a value of “1” and untreated wells a value of “0”. Both peptides were non-toxic to cells at the concentrations tested.

### 3.4.5 Cell Supernatant Calcein Assay

HeLa cells were plated in complete growth media in 96-well plates at 10,000 cells/well and allowed to adhere for 2 days. Peptide treatments were prepared in MEM or OptiMEM at specified concentrations. On the day of treatment, growth media was removed by vacuum and cells were washed 1X with PBS. 200 µL of each treatment was added in triplicate and allowed to incubate for 1 – 4 days. After desired incubation time, 175 µL of media was removed from each well and centrifuged at 2000 rpm for 5 min to remove any cellular debris. A 150 µL aliquot of the supernatant was transferred to a clean 96-well plate for Zip-Tip filtration. Zip-tips were first wetted via aspiration 3X with 50:50 ACN:H₂O: 0.1% TFA solution. Packing material was then equilibrated 3X on H₂O: 0.1% TFA after which the entire cell supernatant was aspirated through the tip 10X. Salts were removed by washing the tip 3X with H₂O: 0.1% TFA. After washing, peptides were eluted by aspirating 100 µL 80:20 ACN:H₂O with 0.1% TFA 10X in a clean 96-well plate. Care was taken to not allow the tip to dry throughout the process. After peptides were eluted in the ACN solution, 100 µL of a calcein-copper indicator solution was added such that the final concentrations were: calcein (5 µM), copper (5 µM), HEPES pH 7.4 buffer (20 mM). Fluorescence was read on a plate reader with λ_ex/em = 490/535 nm.

### 3.4.6 Lipid Raft Targeting

HeLa cells were plated on a 35 mm dish with 1.5 coverglass (MatTek Corp.) in 2 mL complete growth medium and allowed to grow to about 50% confluence. On the day of the experiment, growth media was removed and cells were washed 1X with PBS. 5 µM F-SWH-C was dissolved in OptiMEM (0.1% DMSO) and incubated on the cells for
3 h at 37 °C and 5% CO₂. After incubation, media was removed and cells were washed 1X with PBS. Lipid rafts were labeled according to manufacturer’s instructions (Invitrogen). Briefly, cholera toxin subunit B (CT-B) conjugated to AlexaFluor 594 was dissolved in PBS and diluted in chilled complete growth media. This solution was added to the cells and incubated at 4 °C for 10 min. After incubation, cells were washed 2X with PBS. Anti-cholera toxin subunit B rabbit serum was added to chilled complete growth media and incubated with cells for an additional 15 min at 4 °C. Cells were again washed 2x with PBS and 2 mL OptiMEM was added for microscopy. F-SWH-C was visualized using an Argon 488 nm laser for excitation and green emission filter while lipid rafts were visualized with HeNe 594 nm laser with red emission filter. TIRF excitation was performed using red and green excitation/emission filter cubes. No signal was observed when the excitation wavelengths were switched indicating there was no bleed-through between the red and green channels in either instrument.

3.4.7 DMACA-DABCYL Characterization

2 mM stock solutions of DMACA and Fmoc-Lys(DABCYL)-OH were prepared by dissolving the lyophilized powder in DMSO. Stocks were diluted to 5 µM in 3 mL of either 0.1 M Sodium Acetate Buffer pH 4.5 or 20 mM HEPES buffer (no salt) pH 7.4 (0.25% DMSO). Solutions were transferred to a 1 cm fluorescence cuvette and the fluorescence spectrum recorded with 370 nm excitation. The fluorescence of DMACA was similar at both pHs even in the presence of equimolar Fmoc-Lys(DABCYL)-OH. To evaluate the ability of DABCYL to efficiently quench DMACA fluorescence, the pair was conjugated to either end of the SWH peptide to yield the DMACA-SWH-DABCYL peptide sequence DMACA-EVNLDHAHFWADR-K(DABCYL). The lyophilized peptide was dissolved in DMSO and further diluted in each buffer to yield a final concentration
of 5 µM. In this arrangement, FRET is able to occur and DABCYL efficiently quenches the fluorescence of DMACA.

**3.4.8 In vitro Assays of BACE Activity**

5 mM stock solutions of β-MAP, DMACA-SWH-DABCYL, Peptide β-Secretase Inhibitor III (EMD), and small molecule BACE inhibitor Axon 1125 (Axon Medchem) were prepared by dissolving lyophilized powder in DMSO. Inhibitor structures are shown in Figure 56. Substrate stock solutions were diluted to 10 µM (β-MAP) or 4 µM (DMACA-SWH-DABCYL) in 200 µL 0.1 M Sodium Acetate Buffer pH 4.5 (0.2-0.4% DMSO) in triplicate in 96-well plates. Inhibitors were added to a final concentration of 10 µM. Solution was aspirated by pipette 3X to ensure proper mixing. 4 µL purified BACE ectodomain was added to each well and fluorescence was recorded on a plate reader at 37 °C every 10 min using 355 nm and 460 nm excitation/emission filters respectively. Fluorescence data were normalized to a blank buffer solution.

![Figure 56: BACE Inhibitors](image)

**3.4.9 β-MAP Toxicity**

HeLa cells were plated in 2 mL complete growth media in a 24-well plate and allowed to grow to 70% confluence. On the day of the experiment, 1 M HCl was added dropwise to an OptiMEM (Gibco) stock solution to pH 4.5. Varied concentrations of β-MAP were diluted in DMSO, then diluted further into pH 4.5 OptiMEM so the final
concentration of DMSO in each condition was 0.1%. Media was removed and cells were washed 1X with PBS. 1 mL of β-MAP in acidified OptiMEM was added to the cells and allowed to incubate at 37 °C and 5% CO₂ for 10 min. We have determined that incubating HeLa cells in acidified OptiMEM is non-toxic up to about 40 min (Figure 57). Treatments were removed and 1 mL of unmodified pH 7.4 OptiMEM was added to the cells. Each condition was repeated in triplicate. After 24 h incubation, determination of cell viability was performed using CellTiter-Blue (Promega) and cell death using LDH Cytotoxicity Detection Kit (Roche). Both protocols were performed according to manufacturer’s instructions. β-MAP was shown to be non-toxic over the range of concentrations tested (Figure 46).

![Figure 57: HeLa cell viability in pH 4.5 OptiMEM](image)

HeLa cells treated with pH 4.5 OptiMEM for varying lengths of time before media was replaced with pH 7.4 OptiMEM. Viability measured with Cell Titer Blue assay after 24 hours.

**3.4.10 siRNA Knockdown**

Cells were plated in 2 6-well plates and allowed to grow to 50% confluence in complete growth media. 1 day before transfection, the media was replaced with DMEM
containing 10% fetal bovine serum without antibiotics. BACE specific siRNA sequences (Stealth siRNA, Invitrogen) were transfected using Dharmafect I (Thermo) in OptiMEM for 2 days according to manufacturer’s instructions. After transfection, media was replaced with complete growth media. One 6-well plate was designated for western blot as described below while the cells in the second plate were trypsinized, pelleted by centrifugation, and transferred to an 8-well u-slide (Ibidi) for microscopy.

3.4.11 Western Blot

The cells were washed with ice-cold PBS, harvested in PBS, and centrifuged for 1 min. Whole-cell lysate was isolated using RIPA buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 1.5 mM MgCl₂, 1% NP-40, 1 mM EGTA, 10% Glycerol, 50 mM NaF, 2 mM Na₃VO₄, protease inhibitor cocktail 1:200), and protein concentration of whole-cell lysate was determined using Bio-Rad Bradford reagent using bovine serum albumin (BSA) for standard curve production. For each sample, proteins were resolved by SDS-PAGE and transferred to a PVDF membrane (Biorad). The membrane was blocked with 5% milk in Tris-Buffered Saline and Tween 20 (TBST) buffer for 2 h at room temperature and then incubated with primary antibody (1:1000 (8 µL antibody: 8 mL 5% milk) for BACE (Sigma, Anti-BACE N-terminus), and 1:4000 for β-actin (Sigma, Monoclonal Anti-Beta Actin) overnight at 4 ºC. The membrane was washed with TBST buffer for 10 min three times, incubated with HRP conjugated secondary antibody (1:5000 in 5% milk) for 1 hr at room temperature, and washed with TBST. The blot was then developed using ECL reagent.

3.4.12 Cellular BACE Activity Assay

HeLa cells were seeded onto an 8 well u-slide (Ibidi) in 300 µL complete growth media and allowed to grow to 50% confluence. Media was removed and cells were
washed 1X with PBS. 200 nM β-MAP was added in pH 4.5 OptiMEM (0.1% DMSO) with BACE inhibitors and allowed to incubate for 10 min at 37 °C and 5% CO₂. Treatments were removed and replaced with pH 7.4 OptiMEM containing inhibitors. Cells were visualized on a Deltavision Elite microscope with 60x (oil) or 20x (dry) objectives. Microscope incubation chamber was held at 37 °C and cells were blanketed with a 5% CO₂ : 95% air mixture. DAPI excitation (390 nm) and CFP emission (470 nm) filters were used to collect images every 10-20 min for 5-6 h.
4. Cellular Model of Alzheimer’s Disease and Chelator Efficacy

We have demonstrated in Chapter 3 that β-MAP can be cleaved by BACE in a cellular system, albeit at very low concentrations. Even though we have not directly observed the products of the enzymatic reaction in cells, it is a logical assumption that the chelator is being formed on the plasma membrane at a similar location as Aβ. In order to evaluate whether the chelator can rescue cells from AD-like conditions, a suitable cell system must be developed. This chapter describes our cell model for AD, and the efficacy of the peptide chelators tested in that model.

4.1 Background and Significance.

Alzheimer’s Disease is characterized by widespread damage to multiple cell types including neurons, astrocytes, and oligodendrocytes. Therefore, achieving an accurate representation of AD in a cell model comprised of a single cell line may be overly ambitious. Adding excess extracellular stimulants such as Aβ or copper also may not be an accurate portrayal of a diseased state. However, moving directly into an in vivo model would be difficult to interpret given the complexity and interplay of various factors. Therefore, a cell model that mimics at least some of the factors involved in AD can be used. The results of such assays must be carefully considered and the implications not overstated.

SH-SY5Y cells are some of the most widely used cells in which to simulate AD and other neurodegenerative disease conditions. These cells are used as an alternative to primary mesencephalic neurons, which are extremely difficult to obtain and culture. SH-SY5Y cells are a subclone of SK- N-SH cells, which were taken in the 1970’s from a bone marrow biopsy of a neuroblastoma patient.146 The cells possess many characteristics of dopaminergic neurons, exhibiting tyrosine and dopamine-β-hydroxylase enzyme
activity along with the ability to synthesize and take up dopamine and convert it to norepinephrine. They are also able to proliferate for long periods without contamination using general cell culture procedures. As such, SH-SY5Y cells have been used to study the effect of potential Alzheimer’s and Parkinson’s disease therapeutics, including peptide chelators.

There are several drawbacks to using undifferentiated SH-SY5Y cells as a neuronal cell model however. As the cells continuously divide, it is difficult to evaluate over the course of a multi-day experiment whether the xenophile influences the proliferation rate or the rate of cell death. There are multiple phenotype issues as well such as the low expression of dopamine transporters and receptors, varying stages of neuronal development among cells at different maturity levels, and limited catecholamine synthesis. Perhaps most problematic for our prochelator, which can only be converted to chelator in small amounts, is that SH-SY5Y cells exhibit significantly less sensitivity to neurotoxic and neuroprotective agents than primary neurons. This problem is expected to be exacerbated by the relatively low expression level of BACE in lipid rafts of SH-SY5Y cells. Fortunately, because SH-SY5Y cells were derived from immature neoplastic neural crest cells, they exhibit some properties of stem cells and can be differentiated into a more representative neuronal cell model.

There are multiple agents capable of inducing SH-SY5Y differentiation, the choice of which is dependent on the desired properties of the resultant cell line. These agents include retinoic acid and brain-derived neurotrophic factor for cholinergic neurons, and phorbol ester or staurosporine for a more adrenergic phenotype. Because loss of cholinergic neurons is typical in AD, hence the reason most FDA-approved Alzheimer’s drugs are cholinesterase inhibitors, a more cholinergic phenotype is
desirable to mimic AD conditions. Thus, SH-SY5Y cells differentiated with retinoic acid are becoming an increasingly popular cell system in which to study Aβ toxicity.\footnote{50}

Retinoic acid binds to retinoic acid receptors (RAR’s), which can then heterodimerize with retinoic X receptors (RXR’s) activating a response element (RARE) that results in transcription activation.\footnote{51} The regulation of various pathways by retinoic acid results in several interesting phenotype changes in SH-SY5Y cells. The most obvious being cell growth arrest with extensive neurite outgrowth from each cell. Higher expression of choline acetyl transferase and vesicular monoamine transporter to go along with a decrease in neuropeptide tyrosine all suggest a more cholinergic phenotype for retinoic acid differentiated SH-SY5Y cells.\footnote{47} Particularly advantageous towards our goals, is the enhanced susceptibility of differentiated SH-SY5Y cells towards neuroprotective and neurotoxic agents as compared to undifferentiated cells. These features render retinoic acid differentiated SH-SY5Y cells a desirable cell line in which to test our prochelator strategy.

One major potential drawback to differentiating cells with retinoic acid is the effect it has on the secretases, particularly BACE. In order for our prochelator strategy to be effective, we desire a sensitive cell model with at least comparable BACE activity to HeLa cells. Differentiation with retinoic acid has an antiamyloidogenic effect at the transcriptional, translational, and activation levels. This means that γ-secretase activity is upregulated while β-secretase activity is decreased. The reduction in BACE activity is achieved through a complex sequence of events involving protein kinase C activation, which results in impaired BACE trafficking and a reduction in membrane localization. A lack of BACE activity could obviously have catastrophic effects on the ability of our prochelator to mediate copper-induced toxicity in this cell line.
Possibly the most representative cell model for AD is MC65 cells, which were also derived from a human neuroblastoma cell line (SK-N-MC). These cells have been transfected with an APP construct composed of an N-terminal signal peptide fused with the C99 fragment of APP. This fragment corresponds to the β-cleaved C-terminus of APP, whereby the cell is effectively expressing APP that has already begun amyloidogenic processing. When the protein synthesis inhibitor, tetracycline, is removed from the culture media, the cells express this construct within a couple hours. Endogenous γ-secretase then processes the APP fragment creating Aβ peptides. The accumulation of intracellular Aβ results in measurable cell death within 12 hours and complete death of the colony within 72 hours. The mechanism of apoptotic cell death has been linked to oxidative stress as treatment with antioxidants has proven to be the most effective method for preserving cell viability. These cells also show a slightly enhanced sensitivity towards copper though the effect is not particularly noteworthy. (Personal correspondence with R. L. Woltjer). Unfortunately, these cells are extremely difficult to culture, cannot be passed more than 20 times, and are not commercially available, making a collaboration with Dr. Woltjer necessary to evaluate our prochelator approach. While a collaboration is available, encouraging results with other, more general, cell lines would need to be established to necessitate moving in this direction.

Here we show the effect of treating HeLa, undifferentiated SH-SY5Y, and differentiated SH-SY5Y cells with Aβ and copper under a variety of conditions. A standardized method is developed with which to evaluate various chelators and prochelators under AD-like conditions. We demonstrate the efficacy of CP and cholesterol-anchored CP and discuss the inherent problems with a β-secretase activated prochelator in a cellular model and some approaches to overcoming these problems.
4.2 Results and Discussion

4.2.1 Aβ Toxicity in HeLa Cells

We have demonstrated with β-MAP that the SWH prochelator sequence can be hydrolyzed by BACE to presumably create CP in HeLa cells. Even though this not a neuronal cell line, HeLa cells are easy to culture and express appreciable levels of BACE. We therefore used HeLa cells as a starting point to study the extent of Aβ and copper toxicity. Figure 58 shows the results of HeLa cells treated with various combinations of Aβ and copper.

![Image of bar graph showing Aβ and copper toxicity in HeLa cells]

Figure 58: Aβ and copper toxicity in HeLa cells

HeLa cells treated with Aβ (0-40 μM), CuCl₂ (0-40 μM) and Aβ(Cu) (0-40 μM) for 24 hours. All cells, except OptiMEM, treated with 300 μM ascorbic acid. Cell viability measured with Cell Titer Blue assay. The pH of the conditions marked with an (*) were slightly basic due to high Aβ stock additions to the media. Cell viability measurements at these conditions are likely not representative of only Aβ and Aβ(Cu)-induced toxicity.
All conditions with the exception of OptiMEM contained 300 µM ascorbate to catalyze copper redox cycling and initiate oxidative stress reactions. While at first this data looks promising, it was later discovered that the pH of the media was made slightly basic by the high volume of Aβ stock added at 40 µM conditions. It is necessary to dissolve Aβ in pH 10 water to monomerize and solubilize the peptide. Direct dilution of the basic stock solution into OptiMEM partially overwhelmed the buffering capacity of the media, and created an inhospitable environment for cell viability. Despite pH concerns in this experiment, it was observed that Aβ(Cu) induced significantly more death than Aβ alone where the pH of treatments are the same. This indicates that treating with Aβ, Cu, and ascorbic acid is the most lethal combination to HeLa cells. To address pH variability, the pH of the Aβ stock was adjusted to pH 7.4 with HEPES buffer directly before addition to cells in future experiments. This adjustment was not part of the original protocol because Aβ cannot be stored for long periods of time (> 1h) at neutral pH. It begins to aggregate and precipitate from solution as seen in the turbidity studies in Chapter 2. The HeLa cell studies presented above were not re-evaluated with this modified procedure because HeLa cells were ultimately abandoned as a cell model of AD.

Another piece of valuable information that can be drawn from Figure 58 is that neither Aβ, copper, nor the combination of the two, is very toxic at 10 to 20 µM. At these dosage amounts, the pH did not significantly deviate from 7.4. The lack of toxicity is troublesome for our prochelator strategy because we can only activate nanomolar amounts of chelator at the cell membrane. Since we are not seeing toxicity of the Aβ(Cu) complex until much higher concentrations, it is unlikely that a chelator displacing extremely substiochiometric amounts of copper from Aβ would greatly impact viability. Therefore HeLa cells were abandoned for toxicity studies in favor of the more neuronal-
like SH-SY5Y cells in hopes of gaining enhanced sensitivity to Aβ(Cu) induced stress and to better represent cells affected by AD.

**4.2.2 Aβ Toxicity in SH-SY5Y Cells**

Undifferentiated SH-SY5Y cells are a neuroblastoma cell line available from the ATCC that are commonly used to study the effects of Aβ on cell viability. We cultured these cells as well to obtain a more representative cell model of AD. Upon treatment with various amounts of the Aβ(Cu) complex, we observed a dose-dependent decrease in the cell viability (Figure 59). Care was taken to ensure the pH remained constant throughout the experiment. Additionally, several control experiments were also performed including treating cells with only vehicle as well as Aβ in the absence of copper. None of these control experiments resulted in significant toxicity to cells.
Undifferentiated SH-SY5Y cells treated with Aβ(Cu) (0-40 µM) for 24 hours. All cells treated with 300 µM ascorbic acid. Cell viability measured with Cell Titer Blue assay. 1% Triton X-100 (TX-100) used as negative control.

While these results are consistent with those previously reported in the literature, SH-SY5Y cells remain fairly insensitive to low concentrations of Aβ(Cu). However, like HeLa cells, SH-SY5Y cells are a cancer cell line evolved to be robust under stress. In order to gain enhanced neurotoxicity from Aβ(Cu) and neuroprotection from CP, an even more neuron-like cell model needs to be employed.

### 4.2.3 Aβ Toxicity in Differentiated SH-SY5Y Cells

SH-SY5Y cells can be differentiated into a more cholinergic neuronal cell by 8-day treatment with trans-retinoic acid. Figure 60 shows SH-SY5Y cells cultured in
growth media (left) and growth media with 10 μM retinoic acid (right) for 8 days. The most noticeable phenotype is the presence of multiple neurite extensions connecting nearby cells in the retinoic acid treated cells. No additional characterization on these cells was performed in our lab, however literature reports suggest the phenotype closely resembles that of cholinergic neurons.\textsuperscript{147}

**Figure 60: SH-SY5Y cells differentiated in retinoic acid**

Differentiated SH-SY5Y cells show an increased sensitivity to copper and Aβ(Cu) induced stress. In comparison to undifferentiated cells, which were about 90% viable when treated with 20 μM Aβ(Cu), differentiated SH-SY5Y cells are only 50% viable when subjected to the same treatment (Figure 61). Even though more sensitivity is desirable, various peptide chelators were tested in this model to establish copper chelation with a peptide chelator as a viable approach towards mediating toxicity.
Figure 61: Aβ(Cu) toxicity in differentiated SH-SY5Y cells

8-day retinoic acid differentiated SH-SY5Y cells treated with CuCl₂ (0-30 µM) and Aβ(Cu) (0-30 µM) for 24 hours. All cells treated with 300 µM ascorbic acid. Cell viability measured with Cell Titer Blue assay. 1% Triton X-100 (TX-100) used as negative control.

4.2.4 Chelator efficacy in Differentiated SH-SY5Y Cell Model

4.2.4.1 Rescue by CP

The first chelator to be tested in the differentiated SH-SY5Y AD cell model was the native CP chelator described in Chapter 2. The results presented in Figure 62 are very promising. Stoichiometric amounts of CP completely alleviated the toxicity of the Aβ(Cu) complex.

---

1 Undergraduate student Justin Torosian greatly assisted in evaluating the efficacy of chelators in the AD cell model.
Figure 62: Protective effects of CP against Aβ(Cu) toxicity in differentiated SH-SY5Y cells

8-day retinoic acid differentiated SH-SY5Y cells treated with Aβ (20 μM), CuCl₂ (20 μM) Aβ(Cu) (20 μM), and CP (20, 40 μM) for 24 hours. All cells treated with 300 μM ascorbic acid. Cell viability measured with Cell Titer Blue assay. 1% Triton X-100 (TX-100) used as negative control. CP efficiently protects cells from Aβ(Cu)-induced stress.

The rescue effect is presumed to be a result of the displacement of copper from Aβ and subsequent inhibition of redox cycling that has been demonstrated in vitro. However, there must be additional factors playing roles in cell toxicity as it was observed that neither Aβ, nor copper, were toxic to cells alone. We would expect that if the toxicity was solely a result of redox cycling, then free copper would be equally, if not more toxic than the Aβ(Cu) complex. As this is not the case, we hypothesize that Aβ is acting, at least in part, as a targeting vector that brings copper in contact with cells. This theory is consistent with literature reports that suggest Aβ specifically targets membrane
lipids. Free copper on the other hand, also catalyzes oxidative stress, but it does so in the extracellular media where the ROS is quickly quenched by media components such as amino acids and does not greatly affect cell viability. It is difficult to determine whether exogenous Aβ(Cu) is taken up by cells and exerts its toxic effects intracellularly, or whether the lipophilic tail of Aβ simply embeds in the cell membrane, and with copper, catalyzes membrane damage. We cannot draw a conclusion regarding the toxicity mechanism from this experiment because CP was added at the same time as Aβ(Cu) and displaces the metal very quickly, likely before any interaction with the cell occurs.

Additional experiments in which Aβ(Cu) is allowed to pre-incubate with cells before CP addition could possibly provide insight into which of the two mechanisms results in cytotoxicity. CP is not membrane permeable and would not be able to rescue cells from Aβ(Cu) if the complex was internalized and exerted intracellular damage. However, if the complex catalyzed oxidative stress on the cell membrane, CP may be able to alleviate this ROS and prevent cell death.

Substoichiometric amounts of CP were also used to evaluate the potency of the chelator. In order for our BACE-activated prochelator strategy to be viable, substoichiometric amounts of chelator will need to display significant protective effects against cytotoxicity. Interestingly, as seen from Figure 63, only half an equivalent (10 \( \mu \)M) of CP is necessary to induce full rescue. This is a curious result because it is not intuitively obvious how displacement of only half an equivalent of copper could fully protect cells.

It is possible that the aggregation state of Aβ(Cu) could be playing a significant role. It is well accepted that soluble Aβ oligomers are more toxic than higher order structures such as aggregates or fibrals. CP may be able to more quickly displace
copper from these oligomers based on solution availability as compared to insoluble aggregates. As the nature of exogenous Aβ aggregates that are added to cells in vitro is still highly controversial, we hypothesize that substoichiometric amounts of CP are targeting copper bound to the more toxic oligomers as opposed to precipitated aggregates. This differential targeting may result in a significant protective effect, even at substoichiometric concentrations. These hypotheses await experimental validation and represent avenues of future study.

**Figure 63: Protective effects of substiochiometric CP against Aβ(Cu) toxicity in differentiated SH-SY5Y cells**

8-day retinoic acid differentiated SH-SY5Y cells treated with Aβ(Cu) (20 µM), and CP (5-20 µM) for 24 hours. All cells treated with 300 µM ascorbic acid. Cell viability measured with Cell Titer Blue assay. 1% Triton X-100 (TX-100) used as negative control.
4.2.4.2 Rescue by Cholesterol-anchored CP

CP modified with a cholesterol anchor and PEG linker (termed CPRC) was also evaluated in differentiated SH-SY5Y cells for its ability to protect against Aβ(Cu) toxicity. CPRC (Figure 64) is the chelator product resulting from BACE hydrolysis of the prochelator in cells.

![Structure of CPRC](image)

**Figure 64: Structure of CPRC**

Disappointingly, CPRC is inherently toxic to differentiated SH-SY5Y cells. Figure 65 shows a steady decrease in cell viability with increasing CPRC concentration, unlike that observed with CP. Notably, the toxicity of 20 μM CPRC is about that of 20 μM Aβ(Cu). Obviously this result severely impacts the ability of CPRC to rescue cells from AD-like conditions. Control experiments in which differentiated cells were treated with cholesterol alone did not induce the same toxicity (not shown). We hypothesized that CPRC on the membrane may be preventing the cells from obtaining copper and thus inducing a copper deficiency. An initial attempt to acetylate the N-terminus of CPRC, which would effectively block copper binding, was unsuccessful. Alternative approaches towards studying the toxicity mechanism include substituting the histidine in CPRC for alanine, which would also prevent copper binding or simply using the full SW sequence (EVNLDAEFWADR) with a cholesterol anchor. Investigating the mode of toxicity of CPRC represents another interesting avenue for future research.
Figure 65: CPRC toxicity in differentiated SH-SY5Y cells

8-day retinoic acid differentiated SH-SY5Y cells treated with CPRC in 0.1% DMSO (5-40 µM) for 24 hours. Cell viability measured with Cell Titer Blue assay. 1% Triton X-100 (TX-100) used as negative control.

The toxicity profile could be greatly mitigated if CPRC was not incubated with cells for 24 hours. Rather, if differentiated SH-SY5Y cells were treated for only 10 min with CPRC, analogous to β-MAP, the cytotoxicity of 20 µM CPRC can be reduced from 50% to only 20%. Therefore, a procedure involving pre-treating cells with CPRC for 10 min followed by 24-hour Aβ(Cu) incubations was adapted to evaluate the potency of CPRC. As can be seen from Figure 66, the potency profile is similar to that of CP in that only half an equivalent of chelator (10 µM) is necessary to observe the full protective effect. However, the ability of CPRC to rescue cells from Aβ(Cu) toxicity is very modest. This stems largely from the fact that CPRC is only slightly less toxic than Aβ(Cu) itself.
Figure 66: Protective effects of CPRC against Aβ(Cu) toxicity in differentiated SH-SY5Y cells

8-day retinoic acid differentiated SH-SY5Y cells treated for 10 min with CPRC (0-20 μM) followed by addition of Aβ(Cu) (20 μM) for 24 hours. All cells treated with 300 μM ascorbic acid. Cell viability measured with Cell Titer Blue assay. 1% Triton X-100 (TX-100) used as negative control.

This experiment represents a “best case scenario” for our BACE-activated prochelator strategy in which all of the prochelator is converted to its associated chelator product. Unfortunately, treatment with 5 μM CPRC does not significantly improve cell viability, and in order to have BACE activate the prochelator in cells, an even smaller concentration than this must be used (200 nM). Given the sensitivity limitations of the cell model and the inherent toxicity of CPRC at low concentrations, neither the full cellular prochelator, nor β-MAP were evaluated for their ability to rescue cells from Aβ(Cu) toxicity. However, as has been demonstrated with CP, chelators can have a
significantly positive effect on cell viability in this model. If enough chelator product can be generated from a suitable prochelator, then our original hypothesis could provide a measurable therapeutic benefit. Alterations to the CPRC peptide sequence, targeting vector, or perhaps a different enzymatic activation process could achieve these goals, and represent exciting avenues of future study.

4.3 Conclusions

We have developed a fairly sensitive cell model for AD-like conditions using SH-SY5Y cells differentiated for 8 days with retinoic acid. Treatment with 20 µM Aβ(Cu) complex and ascorbic acid induces 50-60% cell death whereas treatment with any of these compounds individually does not achieve the same degree of cytotoxicity. We have also shown that CP can rescue cells from this toxicity, and can do so at substiochiometric levels. We hypothesized that targeting CP to raft domains through the use of a cholesterol anchor would further improve the efficacy by directing the chelator to a purported site of toxicity. This was not observed to be the case however, as cholesterol targeting imparted cytotoxicity of its own. We were able to show modest rescue from AD-like conditions with the CPRC chelator, but the result was not very significant. Furthermore, the lowest concentration of chelator that could achieve any cell rescue was 10 µM. This is about 50 times higher than the maximum amount of chelator generated using β-MAP. Therefore, even though we did not directly test the full prochelator in cells, we would not expect to see any efficacy at 200 nM, the concentration at which β-MAP was tested.

Overall, we concluded that while the current prochelator construction (peptide-PEG-membrane anchor) does effectively target the prochelator to cell membranes and can be hydrolyzed by BACE, the inherent toxicity and limited loading prevent the prochelator from rescuing cells from AD-like conditions. This construct however, does
provide a suitable platform in which to study BACE hydrolysis in cells. Though not our original intention, β-MAP has multiple academic and industrial applications and could play a significant role in our understanding of AD and the development of therapeutics in the future. Similarly significant is that a prochelator approach towards temperament of AD remains a viable strategy if the amount of chelator generated is therapeutically relevant. If in fact the toxicity of CPRC were related to copper depletion, then we would expect the full prochelator to be significantly less cytotoxic. Furthermore, implementation of the Merck peptide sequence discussed in Chapter 2 could increase the amount of prochelator that can be loaded to the cell membrane and still retain BACE specific hydrolysis. Increased loading and BACE hydrolysis increases the amount of chelator on the membrane, and may enhance its protective abilities. These are a few of the possible future experiments toward developing a BACE activated prochelator for the treatment of Alzheimer’s Disease.
4.4 Experimental

4.4.1 Materials and Instrumentation

All chemicals and solvents were obtained from Sigma-Aldrich and used without further purification unless otherwise noted. All water was nanopure. Peptides were synthesized on a Protein Technologies PS3 automated peptide synthesizer and purified on a Waters 600 HPLC. Liquid chromatography-electrospray mass spectrometry (LC-MS) data were collected on an Agilent 1100 Series HPLC in line with a LC/MSD trap and a Daly conversion dynode detector. Cell titer assays were analyzed on a Perkin Elmer Victor 1420 plate reader.

4.4.2 Peptide Synthesis

The precursor compound Fmoc-Asp(dihydrocholesterol)-OH was synthesized by esterification of the side chain of Fmoc-Asp-OtBu (Novabiochem) with 5α-Cholestan-3β-ol using MSNT / N-Methylimidazole in dichloromethane. Coupling was performed with dry solvents under argon. Deprotection with TFA yielded the Fmoc-Asp(Chol)-COOH building block. Fmoc-NH-(Peg)4-COOH (Novabiochem) was dissolved in DMF with sonication before use.

Peptides were synthesized in 0.1 mmol scale on PAL-PEG-PS resin (Applied Biosystems). Standard Fmoc (9-fluorenlymethoxy-carbonyl)-protected natural and non-natural amino acids (Chem-Impex and Novabiochem) were coupled in 20 min cycles with HBTU (O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate) (Novabiochem) and N-methylmorpholine (NMM) (Acros) in N,N'-dimethylformamide (DMF) (Caledon). Fmoc protecting groups were removed by using 20% piperidine in DMF. Cleavage from the resin and removal of side chain protecting groups was accomplished by treating resin with a 10-mL mixture of 95% trifluoroacetic acid (TFA)
and 2.5% triisopropylsilane (TIS) under nitrogen while shaking for 4 h. Peptide was precipitated from solution by evaporating off TFA with a nitrogen stream, followed by three washes with diethyl ether (Caledon). Purification was accomplished by semi-preparative reversed-phase HPLC on a Waters Spherisorb S10 C8 column with a linear 40 min gradient from 7 to 70% acetonitrile in water with 0.1% TFA. Purity was validated to be greater than 90% by analytical HPLC. Mass of each peptide was determined by ESI-MS. Peptides were stored as lyophilized powder at -20°C until use.

4.4.3 Aβ Preparation

The Aβ1-40 peptide was purchased from EZBioLab and validated by the supplier to be > 95% pure (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV). A portion (1 mg) of Aβ was dissolved in 500 µL of 1% ammonium hydroxide in de-ionized water by sonicating 1 min on, 30 s off, and 1 min on. The solution was filtered through a syringe filter (GE Water, Nylon 0.22 µM, 3 mm). A second aliquot of 250 µL 1% ammonium hydroxide was used to rinse the vial and filter of any residual peptide. The concentration of this stock solution was determined by measuring the tyrosinate absorption (295 nm, ε = 2480 M⁻¹cm⁻¹) at pH 12. Immediately before use, 250 µL of 100mM HEPES buffer pH 7.4 was added to the solution to neutralize the pH and the concentration was recalculated. All solutions were used on the day of preparation.

4.4.4 Cell Culture

Human cervical cancer cells, HeLa cells, were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate at 37 °C and 5% CO₂.

SH-SY5Y cells were cultured in 50:50 Hams F-12 (Gibco): MEM with phenol red (Sigma) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL
streptomycin sulfate, and 1% non-essential amino acids (Gibco). Cells were stored in a HERAcell incubator at 37 °C and 5% CO₂.

### 4.4.5 SH-SY5Y Differentiation

SH-SY5Y cells were plated in 24-well plates and allowed to grow to 30% confluence. Retinoic acid was dissolved in 99% ethanol (190 proof) and 1% water to a final concentration of 10 mM and stored in the freezer protected from light. To initiate differentiation, growth media was removed from the cells and replaced with growth media containing 0.1% retinoic acid (10 µM). After 3-day incubation, cell proliferation had slowed and neurite extensions were visible from the cells. After 4 days, the media was replaced with fresh media containing 0.1% retinoic acid and allowed to incubate for an additional 4 days. Cells were fully differentiated and ready for treatment at ~70% confluence after 8 days.

### 4.4.6 Cell Treatment

HeLa or SH-SY5Y cells were plated to 70-90% confluence (after differentiation for SH-SY5Y cells). Before treatment, Aβ stock concentrations were determined and adjusted to pH 7.4 with HEPES buffer. An aliquot of Aβ stock was removed, combined with an equimolar amount of CuCl₂, and allowed to incubate for 10 min to create an Aβ(Cu) stock solution. CuCl₂ stock solution was sufficiently concentrated so that a very small (<1 µL) sample volume could be added to the Aβ stock and not significantly alter the concentration.

For experiments utilizing CP, treatments were prepared by diluting the appropriate stock solutions in autoclaved eppendorf tubes in the following order with the final concentration listed in parentheses, OptiMEM (total volume 500 µL), CP (0-40 µM), Aβ (10-40 µM), or Aβ(Cu) (10-40 µM). Media was removed and cells were washed
1X with PBS. Treatments were added from eppendorf tubes followed by direct addition of ascorbic acid (300 µM) to each well. For CPRC experiments, cells were pre-incubated for 10 min with CPRC dissolved OptiMEM (0.1% DMSO). CPRC was removed and media was replaced with treatments containing OptiMEM (total volume 500 µL), Aβ (10-40 µM), or Aβ(Cu) (10-40 µM). This was followed by direct addition of ascorbic acid (300 µM) to each well. All treatments were incubated for 24 hours before viability determination by Cell Titer Blue assay.

**4.4.7 Cell Titer Viability Assay**

After 24-hour incubation with treatment conditions, 100 µL Cell Titer Blue was added to each well and allowed to incubate for 4 hours or until sufficient color change was noted. Fluorescence data was recorded on the plate reader (λ_{ex/em} = 560/590 nm) and data was normalized to “1” for untreated cells and “0” for cells treated with 1% Triton X-100. Error bars represent the standard deviation from 3 independent wells.
Appendix A: Characterization of Peptides via LC-MS

Total Ion Chromatogram
Y-axis: Ion Count

UV-Vis Chromatogram
Y-axis: Miliabs
X-axis: Time (min)

Mass Spectrum of largest peak in Total Ion Chromatogram
Y-axis: Ion Count
X-axis: mass/charge

Figure 67: SWH

Peptide structure

Peptide Sequence (single letter abbrev.)
SWH Sequence: EVNLDAHFWADR

Anticipated mass/charge ratio of purified peptide
Expected (m/z) M+H = 1513.7

Ions observed in mass spectrum related to peptide. Ions identified according to protonation state
Observed M+H: 1514.2, M+2H: 758.0

All LC-MS chromatograms in Appendix A follow the same formatting detailed in this example. All analyses performed on Agilent HPLC with in line mass spectrometer. Mobile Phase A: H$_2$O:ACN (98:2) 0.3% Formic Acid. Mobile Phase B: H$_2$O:ACN (2:98) 0.3% Formic Acid. Peptides without cholesterol analyzed on Varian C18, 150 mm column. Peptides with cholesterol anchor incorporated into the sequence analyzed on Agilent XDB-C8, 50 mm column.
Figure 68: CP

CP Sequence: H$_2$N-DAHFWADR

Expected (m/z) M+H: 1016.5

Observed M+H: 1016.6, M+2H: 508.9

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 69: SW

SW Sequence: EVNLDAEFWHDR

Expected (m/z) M+H: 1571.7

Observed M+H: 1571.9, M+2H 786.6

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 70: SW CP

SW Cleavage Product (CP) Sequence: DAEFWHDR

Expected (m/z) M+H: 1074.5

Observed M+H: 1074.5, M+2H: 537.8

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 71: Peptide OK

Peptide OK Sequence: EIDLMVLDWHDR

Expected (m/z) M+H: 1582.8

Observed: M+H: 1583.0, M+2H: 792.2

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 72: OK-SSH

OK-SSH Sequence: EIDLSSHDWADR

Expected (m/z) M+H: 1484.7

Observed M+H: 1484.9, M+2H: 743.1

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 73: SW-SSH

SW-SSH Sequence: EVNLSSHDWADR

Expected (m/z) M+H: 1469.7

Observed M+H: 1469.9, M+2H: 735.7

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Proteomics 1 Sequence: DETLDAHFW

Expected (m/z) M+H: 1174.5

Observed: M+H 1174.7, M+2H: 587.7

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 75: Proteomics 2

Proteomics 2 Sequence: APSLDAHFW

Expected (m/z) M+H: 1084.5

Observed M+H: 1084.6

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 76: Proteomics 3

Proteomics 3 Sequence: ASNLDHAHFW

Expected (m/z) M+H: 1101.5

Observed: M+H: 1101.7

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 77: Kyoto

Kyoto Sequence: El(Thi)(Thi)(Nva)AHFRW

Expected (m/z) M+H: 1404.6

Observed M+2H: 703.1

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 78: Merck

NFEV Sequence: EVNFEVEFRW

Expected (m/z) M+H: 1395.6

Observed M+H: 1396.3, M+2H: 698.5

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 79: F-SWH

F-SWH Sequence: Fluorescein-EVNLDADHFWARD

Expected (m/z) M+H: 1829.8

Observed M+2H: 915.7, M+3H: 610.8

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 80: F-SWH-C

F-SWH-C Sequence: Fluorescein-EVNLDADFWADR-D(Cholesterol)

Expected (m/z) M+H: 2315.1

Observed M+2H: 1158.7, M+3H 772.7

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 81: C-term

C-term Sequence: EVNLD AHFWADR-D(Cholesterol)

Expected (m/z) M+H: 1999.1

Observed M+H: 2000.0, M+2H: 1000.5

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 82: N-term

N-term Sequence: D(Cholesterol)-SEVNLDHFWADR

Expected (m/z) M+H: 2086.1

Observed M+H: 2087.1, M+2H: 1044.1

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 83: MCA-SWH-DNP

MCA-SWH-DNP Sequence: 7-Methoxycoumarin-EVNLDAHFWADR-K(dinitrophenyl)

Expected (m/z) M+H: 1981.8

Observed M+H: 1981.9, M+2H: 991.8

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
**Figure 84: Rho-SWH-Fluor**

Rho-SWH-Fluor Sequence: Rhodamine-EVNLDHFWADR-K(Fluorescein)

Expected (m/z) M: 2459.1 (parent ion is positively charged)

Observed M+2H: 820.2, M+3H: 615.4

Note: Two peaks observed due to mixture of rhodamine isomers. Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 85: DMACA-SWH-DABCYL

DMACA-SWH-DABCYL Sequence: DMACA-EVNLDHFWADR-K(Dabcy1)

Expected (m/z) M+H: 2080.0

Observed M+2H: 1041.0, M+3H: 694.4, M+4H: 521.1

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 86: Scrambled FRET

Scrambled FRET Sequence: DMACA-FVHDNALEWADR-K(Dabcyl)

Expected (m/z) M+H: 2080.0

Observed M+2H 1040.9, M+3H: 694.4, M+4H: 521.0

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 87: FPC

FPC Sequence: DMACA-EVNLDHAHWADRK(Dabcyl)-Peg₄-Peg₄-Peg₃-Peg₄-D(Cholesterol)

Expected (m/z) M+H: 3686.0

Observed M+4H: 922.8, M+5H: 738.4

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 88: $\beta$-MAP

$\beta$-MAP Sequence: DMACA-EVNLDHAHFWADR-K(Dabcyl)-(Peg$_3$)-R-D(Cholesterol)

Expected (m/z) M+H: 3595.0

Observed M+3H: 1199.8, M+4H: 900.0, M+5H: 720.2

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
**Figure 89: Scrambled β-MAP**

β-MAP Sequence: DMACA-FVHDNALEWADR-K(Dabcyl)-(Peg$_3$)-R-
D(Cholesterol)

Expected (m/z) M+H: 3595.0

Observed M+4H: 900.1, M+5H: 720.4

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
CPRC Sequence: DAHWADR-(Peg₄)₃-R-D(Cholesterol)

Expected (m/z) M+H: 2531.5

Observed M+ 2H: 1266.8, M+3H: 844.9, M+4H: 634.0

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
Figure 91: BACE Inhibitor Axon 1125

Expected (m/z) M+H: 579.3
Observd M+H: 579.4

Note: Refer to Figure 67 regarding axis labels, chromatogram identification, ion assignment, and analysis conditions.
References


33. Ye, S.; Huang, Y.; Mullendorff, K.; Dong, L.; Giedt, G.; Meng, E.; Cohen, F.; Kuntz, I.; Weisgraber, K.; Mahley, R., Apolipoprotein (apo) E4 enhances amyloid beta peptide production in cultured neuronal cells: ApoE structure as a potential...


54. Acevedo, K. M.; Hung, Y. H.; Dalziel, A. H.; Li, Q. X.; Laughton, K.; Wikhe, K.; Rembach, A.; Roberts, B.; Masters, C. L.; Bush, A. I.; Camakaris, J., Copper


85. Faller, P., Copper and Zinc Binding to Amyloid-beta: Coordination, Dynamics, Aggregation, Reactivity and Metal-Ion Transfer. *ChemBioChem* 2009, 10 (18), 2837-2845.


Biography

The author was born on August 18, 1983 in Reading, Pennsylvania. In 2001 he graduated from Hamburg Area High School in Hamburg, Pa. He attended Bucknell University in Lewisburg, Pa where he participated in the Sigma Alpha Epsilon fraternity and was a 4-time letter-winner and captain on the Division I Track and Field team. Drew graduated in 2005 with a B.S. in Chemistry with minors in Mathematics and Philosophy and was selected as a member of the National Society of Collegiate Scholars.

In the fall of 2005, Drew began working as a Quality Control Chemist at McNeil Consumer Healthcare in Fort Washington, Pa where he performed various analytical assays on solid dose Tylenol® products and raw materials. He went to Celator Pharmaceuticals in October 2006 as an Analytical Research and Development Associate where he developed new methodology to evaluate novel cancer treatments in Phase I and II clinical trials.

Drew enrolled in the Chemistry Department at Duke University in the fall of 2008 and began working under the direction of Dr. Katherine Franz. During his tenure at Duke, Drew received the Burroughs Wellcome Award and presented his work at various national conferences and universities including his alma mater, Bucknell. His work has resulted in publications in The Journal of the American Chemical Society and Comprehensive Inorganic Chemistry II with several others awaiting submission. Following his graduate work with Dr. Franz, Drew will pursue employment in industry working in early-stage drug development.